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Original Communications

THE ETIOLOGY AND PATHOGENESIS OF FUNNEL CHEST, PIGEON BREAST, AND RELATED DEFORMITIES OF THE ANTERIOR CHEST WALL

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Deformities of the anterior chest wall of congenital or developmental origin have been discussed in the literature for more than 350 years. Nearly all of the articles have dealt with depression deformities, the protrusion deformities having been largely ignored; and, until recent years, the material has been limited to very few cases. Larger series of cases now being reported continue to be concerned with the depression deformities and little or no attempt has been made to classify these. A study of the anterior chest wall deformities of congenital or developmental origin indicates that, from the point of view of etiology and pathogenesis, the depression deformities and the protrusion deformities have much in common and it is with this phase of the subject that this paper deals.[1] The material upon which this is based consists of personal experience with 161 operations in 150 cases (122 depression deformities and 28 protrusion deformities); a considerable number of cases which, for various reasons, did not come to operation; and 6 autopsies seen at Bellevue Hospital through the courtesy of the Department of Pathology.

These deformities fall into three groups, two of which seem to be exact opposites and the third to be a heterogeneous group of cases which have characteristics of both. In the first group are the depression deformities or funnel chests (pectus excavatum). In the second group are the protrusion deformities wherein are the pigeon breasts (pectus carinatum) and various other deformities characterized by protrusion. The third group consists of cases which have physical characteristics of both.

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To prevent ambiguity, funnel chest is defined as a funnel-shaped or conical depression of the anterior chest wall in which the sternum and attached costal cartilages curve backward, the xiphoid is at the apex of the funnel, *and the distance between xiphoid and vertebral column is abnormally short.* These deformities may be symmetrical or asymmetrical; and they may be present at birth or develop later as the child grows, particularly during the rapid growth associated with puberty.

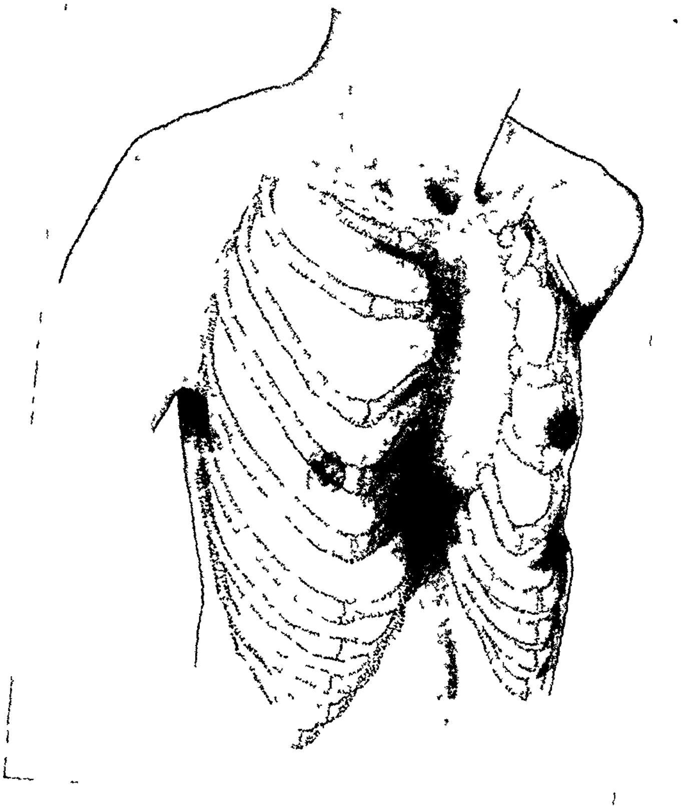


Fig. 1.—Funnel chest deformity of anterior chest wall.

Symmetrical deformities have the same amount of chest wall depression on each side of the midline and there is a transverse groove resembling the Harrison's groove of rickets (Fig. 1). The anteroposterior measurements of the chest are decreased to a greater or less extent thereby producing a caudad obliquity of the anterior ribs which is strikingly apparent on x-ray examination. The heart is displaced into the left chest. In young children, the abdomen has the appearance of a typical pot belly which is produced by the thoracic depression.

The asymmetrical deformity has the xiphoid in the deepest part of the depression as in the symmetrical type but the lateral portion of the deformity is more marked on one side than the other, usually the right. The other features are present but the obliquity of the ribs is greater on the more depressed side.

A characteristic of funnel chest in an infant or child is retraction of the xiphoid during inspiration. This paradoxic motion is almost never associated with dyspnea. When dyspnea is present, the other characteristics of funnel chest are usually absent because the retraction of the xiphoid is the result of the dyspnea and not the cause of it.

In the protrusion type of deformity, the protrusion may be in the midline and involve the sternum or it may be lateral to the sternum and correspond to the costochondral junctions, or in the costal arch. In the midline deformities the sternum may arch forward producing the shape of a pigeon breast (Fig. 2); or it may protrude in an oblique fashion with the xiphoid the most prominent part (Fig. 3). When the protrusion is lateral to the sternum it involves the costochondral rib junctions or the costal arch, which then flares (Fig. 4). The flaring costal arch does not necessarily appear on the same side as the lateral deformity and may be on both sides. A flaring costal arch with the arcuate protrusion deformity simulates funnel chest (Fig. 5). However, there is adequate distance between xiphoid and vertebrae. The protrusion deformities may be present at birth but usually do not appear until the child has had a chance to grow, often not until adolescence. The deformity then seems to progress with growth.

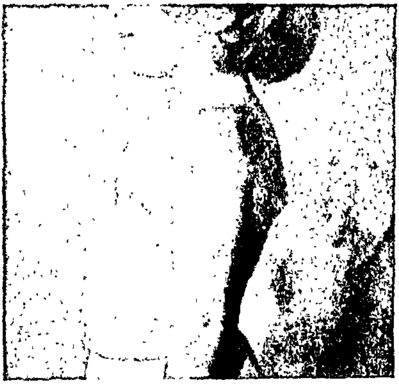


Fig. 2.—Arcuate type of pigeon breast in 6-year-old girl.

Between the depression deformities and the protrusion deformities there is an indefinite group which seems to combine characteristics of both. The lower end of the sternum may be retracted while the upper portion may protrude. There may be an asymmetrical funnel chest with the more prominent side protruding like a lateral protrusion deformity. Occasionally a protrusion in early childhood or infancy may end up as a funnel chest after puberty. The group is, therefore, a heterogeneous mixture but the dominant features of each case tend to place it in one of the two main groups.

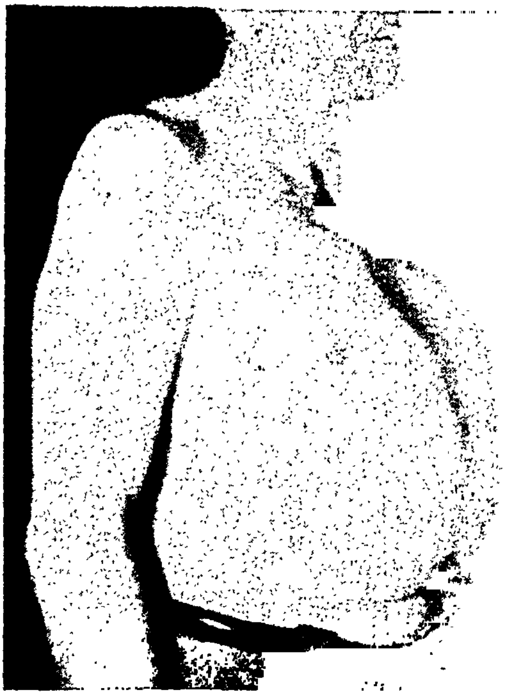


Fig. 3.—Oblique type of pigeon breast in a man 58 years of age. His twin sister has a similar deformity and her son has a funnel chest.

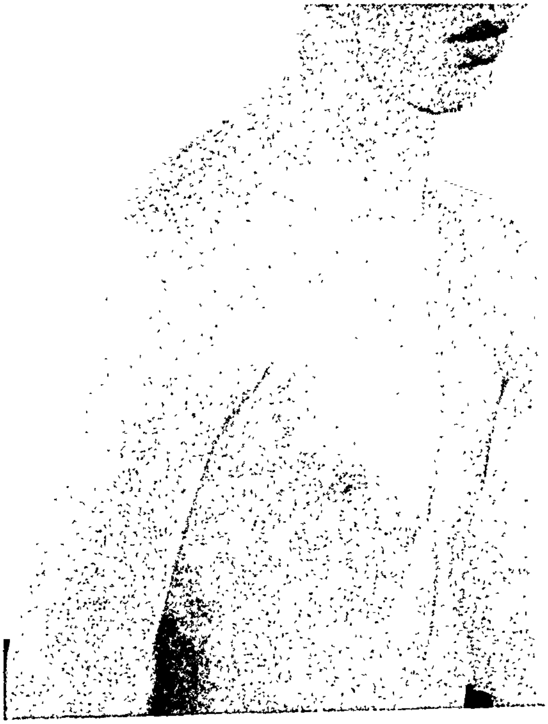


Fig. 4.—Lateral type of protrusion in a 14-year-old boy.

Heredity is mentioned as an etiological factor in funnel chest by nearly every writer on the subject. This factor is also noted in some of the few articles on protrusion deformities. Little has been said, however, of the rather striking fact that both types of deformity can appear in the same family. In the series on which this paper is based, there are numerous instances where one sibling has a funnel chest and another a pigeon breast; or where a parent, an uncle, or an aunt has a pigeon breast and the patient a funnel chest or vice versa. Tilney[2] tells of a pair of dissimilar twins, one with a funnel chest and the other with a pigeon breast. The hereditary factor, therefore, seems to apply to anterior chest wall deformities in general and not specifically to funnel chest or pigeon breast.

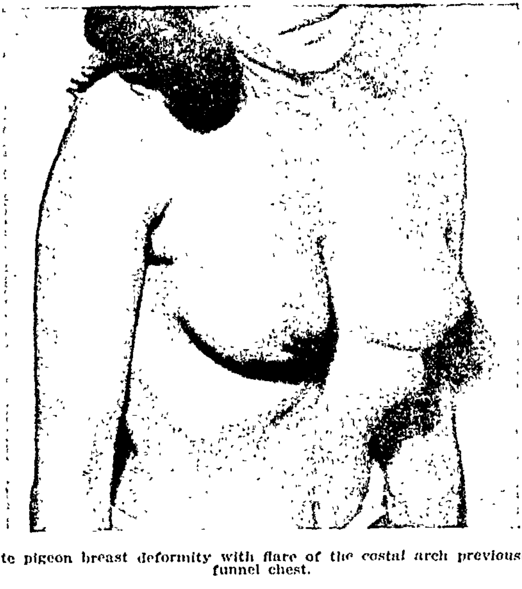


Fig. 5.—Arcuate pigeon breast deformity with flare of the costal arch previously reported[1] as funnel chest.

The anatomy of the thorax and its function as a bellows need not be discussed here except for two features. The first is the substernal ligament which was described by Brown[3] as an extension of the linea alba and the posterior rectus sheath up onto the posterior periosteum of the sternum with which it is intimately fused. Its dynamics are entirely those of the attached muscles.

The second is the diaphragm with its central tendon which is domed when relaxed at the end of expiration and flattened at the end of inspiration. It is an integral part of the chest bellows mechanism and essential in the production of an effective cough.

In a previous paper,[4] it was pointed out that the midline protrusion deformities are caused by proportionate overgrowth of paired ribs. If the overgrowth takes place in the upper ribs they will push the sternum forward as an arch (Fig. 6) and, in so doing, these ribs will buckle at their weakest points, usually the costochondral junctions, and the costal cartilages will slant forward to the elevated sternum. The formation of the arch is aided by the diaphragm which holds the xiphoid in place. If the ribs attached to the lower part of the sternum overgrow, the sternum is protruded in an oblique fashion (Fig. 7). If the diaphragm does not enlarge proportionately with the ribs, the protrusion is made at the expense of the dome of the diaphragm.

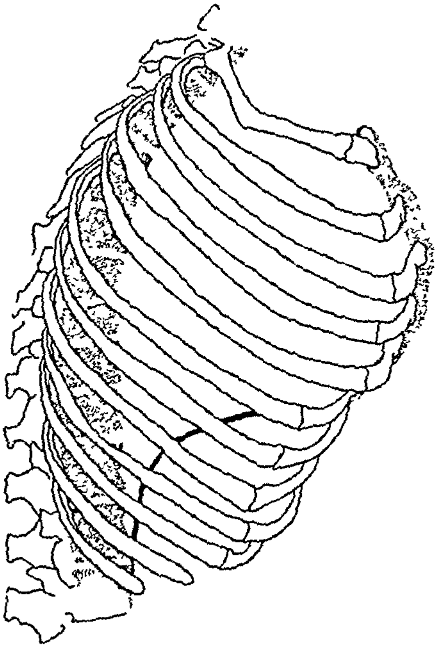


Fig. 6.

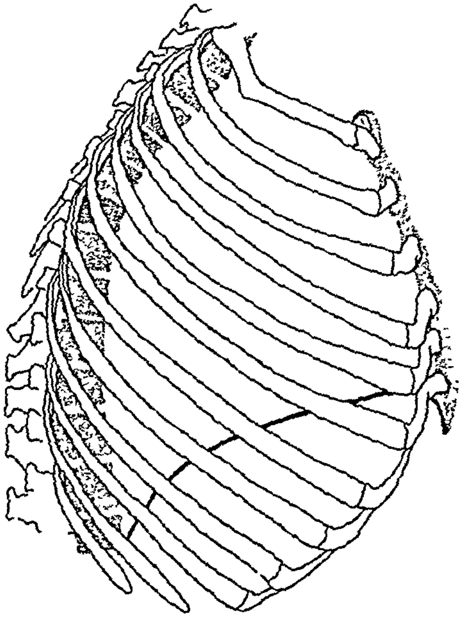


Fig. 7.

Fig. 6.—Arcuate type of protrusion deformity produced by proportionate overgrowth of second, third, and fourth pairs of ribs.

Fig. 7.—Oblique type of protrusion deformity produced by proportionate overgrowth of pairs of ribs attached to lower portion of sternum.

If the ribs above the level of the xiphoid are disproportionately longer on one side only, the ribs will again buckle at the costochondral junctions, but in this case the costochondral junctions will form a protruding ridge lateral to the sternum and their costal cartilages will slant backward to the sternum (Fig. 8). When overgrowth occurs in the ribs below the level of the xiphoid, the costal arch will flare, the diaphragm acting in the same manner on the costal arch that the sternum does on the upper ribs in the arcuate form of sternal protrusion.

Thus, the diaphragm seems to play a role of varying importance in the formation of these deformities. Observations made in the autopsy room tend to support this theory. In the depression, or funnel chest deformities, the diaphragm plays a much more important role.

Funnel chest deformities have a much more extensive literature than the protrusion deformities and this dates back to 1600 when Bauhinus[5] described such a case. Ochsner and DeBakey,[6] in an article published in 1939, list 142 references and since that time many other articles have appeared. Many of the authors speculate on the causes of the deformity but only three of these need be given serious consideration. These are: (1) the pull of a diaphragm with a short central tendon; (2 abnormal growth of the ribs; and (3) obstruction in the respiratory tract above the carina.

Bauhinus[5] believed from his observation that a muscular pull was responsible for the deformity, and the only muscle that can thus retract the sternum is the diaphragm. Woillez,[7] in 1860, also believed that the diaphragm was responsible and predicated that a condition similar to Dupuytron's contracture of the palmar fascia existed in the central tendon. Brown,[3] in 1939, believed that a short central tendon of the diaphragm was responsible and, on this basis, established the principle of the successful surgical treatment of the condition.

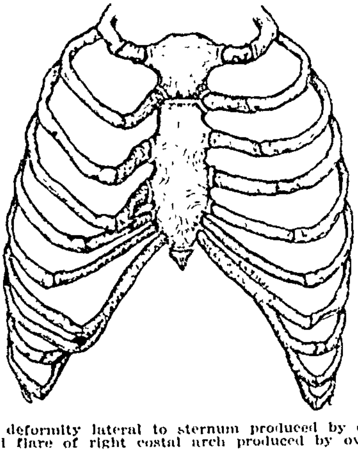


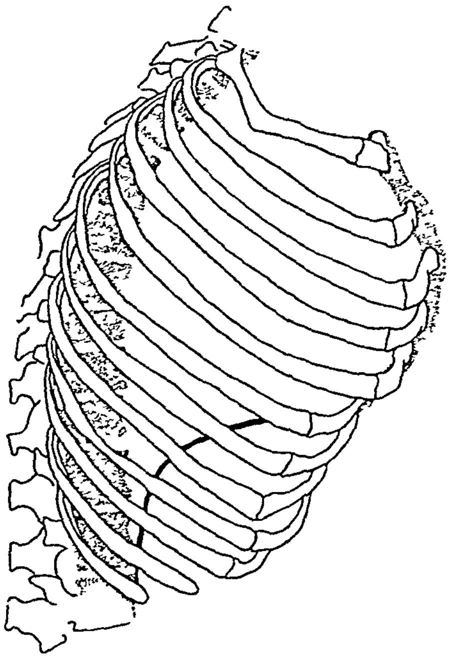
Fig. 8.—Protrusion deformity lateral to sternum produced by overgrowth of ribs 2, 3, and 4 on the right; and flare of right costal arch produced by overgrowth of ribs 7 and 8 on right.

Overgrowth of the ribs was mentioned by Flesch,[8] in 1873, as the cause of funnel chest, an idea which he ascribed to von Schiffer. More recently, Hausmann[9] also believes that this is the cause.

Obstruction in the respiratory tract was thought by Christian,[10] and some of the preceding editors of Osler's textbook, to be a factor in the production of funnel chest because, when this is present, the resulting dyspnea causes a marked retraction of the sternum which has the typical appearance of funnel chest. This will be considered later.

In the group of cases under consideration, observation indicates decidedly that a short central tendon of the diaphragm is the major factor in causing funnel chest. The salient features in support of this follow.

In a previous paper,[4] it was pointed out that the midline protrusion deformities are caused by proportionate overgrowth of paired ribs. If the overgrowth takes place in the upper ribs they will push the sternum forward as an arch (Fig. 6) and, in so doing, these ribs will buckle at their weakest points, usually the costochondral junctions, and the costal cartilages will slant forward to the elevated sternum. The formation of the arch is aided by the diaphragm which holds the xiphoid in place. If the ribs attached to the lower part of the sternum overgrow, the sternum is protruded in an oblique fashion (Fig. 7). If the diaphragm does not enlarge proportionately with the ribs, the protrusion is made at the expense of the dome of the diaphragm.



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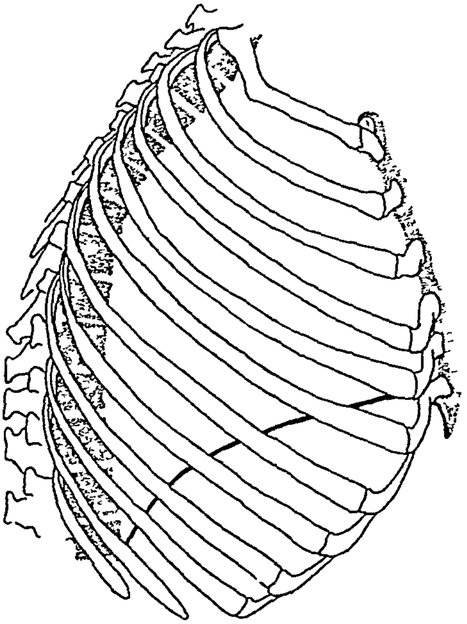

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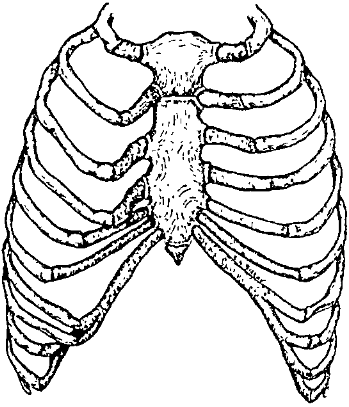

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In the group of cases under consideration, observation indicates decidedly that a short central tendon of the diaphragm is the major factor in causing funnel chest. The salient features in support of this follow.

First: In infants and young children with funnel chest measurement of the anteroposterior diameter of the chest at the xiphoid show it to be retracted during inspiration often as much as 2 cm., while the same measurement in the midclavicular lines show the chest wall to move forward as much or more during inspiration. As children grow older the chest wall tends to lose some of its great resiliency and the retraction of the xiphoid decreases until, in adults, the xiphoid becomes fixed in a backward position while the forward inspiratory movement in the midclavicular line continues.

Second: When the patient is relaxed during sleep, the breathing is often noisy and the retraction greater. With greater relaxation under anesthesia, the retraction is exaggerated and the breathing may become quite labored. The use of an endotracheal tube eliminates the possibility of any obstruction above the carina. During the operation, the breathing difficulty increases as the costal cartilages are resected, often to such an extent that an assistant must exert traction on the xiphoid to restrain the paradoxic motion until the xiphoid is detached and the substernal ligament divided. When the diaphragm is thus released, respiration becomes so much easier and quieter that the anesthetist may become alarmed, fearing that it has ceased.

Third: Until a few years ago some depressions that were well corrected when the patient was discharged from the hospital tended to recur to a certain extent, especially after simple sternoplasty, and in a few instances reoperation was necessary. On the supposition that the regenerated seventh costal cartilage again allowed the diaphragm to exert its pull, the beds of the sixth and seventh cartilages and the intervening intercostal bundles were completely detached from the sternum along with the xiphoid. Since adopting this procedure, the corrections have been much better and the need for reoperation has not arisen.

Fourth: Examination at autopsy of 3 cases of funnel chest, one in a man 62 years of age, one in a woman somewhat younger, and the other in an infant of 2 years, showed that the central tendon of the diaphragm was actually much shortened. In the child, it measured 3 cm. and in the man of 62, only 1.5 cm. In each instance, the diaphragm presented a low dome for each leaf separated by the central tendon, instead of a much higher single dome as in the entire normal diaphragm, and strikingly demonstrates how seriously diaphragmatic respiration is compromised.

From the above, it is evident that the diaphragm is an essential factor in the production of funnel chest deformities, but, that overgrowth of the ribs may be a factor can also be demonstrated. It has just been mentioned that the deformity in some cases of funnel chest was absent or of minor extent in early childhood but developed as the child grew, particularly during the rapid growth of puberty. It is possible to explain this by the factor of rib growth.

In these cases during infancy it can be assumed that the central tendon of the diaphragm is moderately short or lacks the ability to grow. As the ribs lengthen the central tendon does not keep pace with them and the diaphragm exerts an increasing pull on the lower end of the sternum, eventually producing a funnel chest with the same symptoms that obtain when the deformity is present at birth. Anatomically, the chest is not as much flattened nor is the caudad slant of the ribs as great as in the early cases.

Unequal growth of the ribs, as in the lateral protrusion deformities, can explain the asymmetrical funnel chest deformities. A short central tendon is present as in the symmetrical deformities but the ribs grow more on one side than on the other and the depression becomes deeper on the side where the ribs are shorter. As in the case of the protrusion deformities, the asymmetry is absent or minimal during infancy and increases with growth.

In the indefinite group of anterior chest wall deformities, the same factors obtain. A central tendon only slightly shortened in the presence of rib overgrowth can give the appearance of a funnel chest although the xiphoid does not have a paradoxic motion. Carrying this a step farther, there may be an arcuate type of pigeon breast with a definitely shortened central tendon and paradoxic respiration. Two such cases in this series had the symptoms of funnel chest but the arcuate protrusion of the sternum caused the short central tendon to pull the sixth costal cartilages behind the fifth so that, at operation, the fifth costal cartilage on each side had to be resected before the sixth could be approached.

A *third case was essentially one of protrusion deformity with bilateral* flares in the costal arch and an arcuate type of pigeon breast together with a bad round back posture. Release of the diaphragm with correction of the flares and elevation of the lower end of the sternum allowed the child to maintain an erect posture which was not possible before operation.

Another case was followed for five years as a protrusion deformity but, with the accelerated growth of puberty, respiratory difficulty and decreased exercise tolerance appeared and it was then noticed that the diaphragm was moving in a paradoxic manner. The radical operation for funnel chest relieved the symptoms.

Thus, it can be seen that the cases in this heterogeneous group present some clinical characteristics of both protrusion deformities and depression deformities. The symptoms, however, are produced by the dominant factor.

Pseudo-funnel chest is a name which can be given to another group of cases often confused with funnel chest but actually entirely different. The condition is noted at birth or shortly thereafter and is characterized by dyspnea and marked paradoxic motion of the xiphoid. The dyspnea may be the result of pneumonitis from the aspiration of amniotic fluid, a congenital heart lesion, obstruction in the respiratory tract or any other condition which can cause dyspnea in a newborn baby. Forced inspiration with a quick build-up of negative pressure produces the paradoxic motion and not a short central tendon of the diaphragm. The treatment is the treatment of the cause of the dyspnea, but traction on the xiphoid to restrict or prevent the paradoxic motion may bring dramatic relief.

SUMMARY AND CONCLUSIONS

Study of a considerable number of cases indicates that anterior chest wall deformities characterized by depression and those characterized by protrusion can be classified together.

Heredity is a most important etiological factor and either type of deformity can be inherited from the same ancestor.

Size and shape of the diaphragm and disproportionate growth of the ribs are the principal factors in the pathogenesis of these deformities.

If a short central tendon of the diaphragm is the dominant factor, funnel chest develops. If disproportionate overgrowth of the ribs is the dominant factor, one of the types of protrusion deformity will result. When both factors are present in the same individual the dominant deformity will be modified.

Pseudo-funnel chest is an inspiratory retraction of the anterior chest wall in an infant shortly after birth which is due to some pathologic condition in the cardiorespiratory system and not to the factors of true anterior chest wall deformities.

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THE INFLUENCE OF INTRA- AND EXTRATHORACIC OPERATIONS ON THE ELECTROCARDIOGRAM IN PATIENTS WITH PULMONARY TUBERCULOSIS

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THE effect of the various active methods of treatment of pulmonary tuberculosis on the electrocardiogram has been studied by many investigators, and the electrocardiographic changes caused by pneumothorax treatment, phrenic exairesis and, in later years, by thoracoplasty and pulmonary resection, have been described extensively.

Weinshell and associates[15] examined 45 patients and they arrived at the conclusion that practically all electrocardiographic alterations were due to changes in position of the heart. They studied this in pneumothorax, pneumoperitoneum, thoracoplasty, and pneumonectomy. Weiss[16] believed that great importance should be attributed to the position of the mediastinum. When *the mediastinum was displaced to the left, he observed a more vertical position* of the heart; he also ascribed the occurrence of negative T waves in the precordial leads to this displacement. A subclinical cor pulmonale, developing in the postoperative period, was also said to be a factor in the formation of these negative T waves. Thus, changes in the position of the heart, as well as myocardial abnormalities, might be the cause of postoperative electrocardiographic changes. To illustrate this, Weiss cited Biocca and his co-workers[3] who attributed late T changes after lobectomy and pneumonectomy to myocardial damage. During the first few postoperative days, Biocca observed changes in the S-T segments, which entirely resembled the abnormalities found in acute pericarditis.

Armen and Frank[1] likewise observed changes of the T waves, but they believed that these were caused by the presence of air between heart and chest wall. The electrocardiographic abnormalities occurred most frequently after a left pneumothorax treatment. In only 4 out of 68 patients did Hertzman and Mathisen[7] find postoperative electrocardiographic changes which, in their opinion, were not due to the altered position of the heart because these 4 patients showed an isoelectric T wave in the first standard lead.

The same abnormality of the T wave in the first lead was observed by Rauchwerger and Erskine.[12] These authors were unable to find a satisfactory explanation of this phenomenon. Hoppe[8] frequently observed a P pulmonale after pulmonary resection and he saw also transient S-T and T changes.

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The following pages deal with our personal observations of electrocardiographic changes in a large series of patients with pulmonary tuberculosis treated by thoracoplasty or pulmonary resection.

METHODS OF EXAMINATION

In every electrocardiographic examination a great number of leads were taken, namely, the three standard leads, the three unipolar leads according to Goldberger, and the precordial leads according to Wilson from V_1 to V_6, inclusive.

The position of the heart was determined according to Wilson's criteria; in order to avoid micro-electrocardiography the semivertical and the semihorizontal positions were regarded as intermediate.

A preoperative electrocardiogram was made of more than 500 patients selected for operation; special attention was given to the position of the heart. In 444 patients of this group a postoperative electrocardiogram was recorded (at least six months after the operation); this electrocardiogram was compared with the preoperative one.

In a consecutive series of 113 patients of this group, one or more electrocardiograms were also made in the immediate postoperative period. In 28 consecutive patients of the latter group, the arterial oxygen saturation was determined simultaneously with the electrocardiogram. This was done by means of the hemoreflector of Brinkman.[17] The blood was taken from the brachial artery. To augment the accuracy of the determination, the point of maximal saturation as well as the point of maximal desaturation of each blood sample was also determined (Zijlstra[17]).

The presence or absence of pneumothorax was verified in 63 of the 113 patients mentioned by means of x-ray pictures made simultaneously.

RESULTS

There was a remarkably small number of patients in whom electrocardiographic abnormalities were found before the operation; only 12 of the 508 patients were to be regarded as having a pathologically changed electrocardiogram. In 10 patients, the T waves were abnormal; the standard leads, the unipolar as well as the precordial leads showing abnormalities in these waves. T_{III} was negative in all these 10 patients; in 5 of them T_{II} was isoelectric or negative. Also, in 5 patients the T_I was negative or isoelectric. In all patients, T_{aV_F} was pathologic, while 3 patients had a negative T wave in all precordial leads. Less severe T-wave changes in the precordial leads were present in the other patients. In 9 of these 10 patients, the T-wave abnormalities had improved after operation or had entirely disappeared; in one patient they remained unchanged. Two of these 12 patients showed a disturbance of conduction: one a prolonged P-R interval and the other an arborization block.

Table I lists the data of the position of the heart in the 508 patients and the distribution over the various age groups. According to this table, an intermediate position of the heart was found in 51 per cent of the cases, while a horizontal position was observed in only 8 per cent. This should probably be attributed to the fact that the investigation dealt mainly with patients of the younger age groups.

TABLE I

POSITION OF THE HEART	10-19 YEARS	20-29 YEARS	30-39 YEARS	40 YEARS AND OLDER	TOTAL	PERCENTAGE
Intermediate	9	62	44	25	140	28
Intermediate cwr*	10	33	38	16	97	19
Intermediate ccwr†	1	8	10	3	22	4
Vertical	18	38	25	18	99	20
Vertical cwr	7	33	30	11	81	16
Vertical ccwr	3	10	7	5	25	5
Horizontal	-	5	6	5	16	3
Horizontal cwr	-	8	8	12	28	5
Totals	48	197	168	95	508	100

*cwr = clockwise rotation.
†ccwr = counterclockwise rotation.

Comparison of the preoperative and postoperative electrocardiograms of 444 patients proved that there was still a manifest change in position in 94 cases six months after the operation (that is, in 21 per cent of cases).

Table II shows the distribution over the various operations.

TABLE II

TYPE OF OPERATION	TOTAL NUMBER	CHANGE IN THE POSITION OF THE HEART AFTER OPERATION	
		TOTAL	PER CENT
Thoracoplasty	60 } 79	11 } 14	18
(Schede modification)	19 }	3 }	
Pleurectomy	17	3	18
Segmental resection	233	45	19
Lobectomy	78	16	21
Pneumonectomy	37	16	43
Totals	444	94	21
Right-sided operations	208	37	18
Left-sided operations	236	57	24

It is evident that the frequency of change of position was significantly higher after pneumonectomy than after thoracoplasty, segmental resection, or lobectomy. The latter groups show only slight mutual differences. An operation on the left hemithorax led to a somewhat higher percentage of changes of position than an operation on the right side, but this difference is not statistically significant. After a left pneumonectomy there was a tendency to a more horizontal position of the heart. The same was observed after left and right lobectomy. In the other operations, no particular tendency could be distinguished.

Directly after the operation the electrocardiogram usually showed an increase of the frequency combined with a diminution of the amplitude of the QRS complex.

Thirty-five of the 113 patients, who were electrocardiographically examined immediately postoperatively, also had other abnormalities that were to be regarded as pathologic. These abnormalities were always located in the S-T segments and the T waves. In 5 patients, elevated S-T segments were found, namely, in Leads I, II, aV$_L$ and V$_6$ (Fig. 1, *D*). The other patients only showed changes in the T waves; the most frequent observation was negativity of the T waves in all precordial leads. This was the case in 21 patients (Fig. 1, *A*, *B*, and *F*).

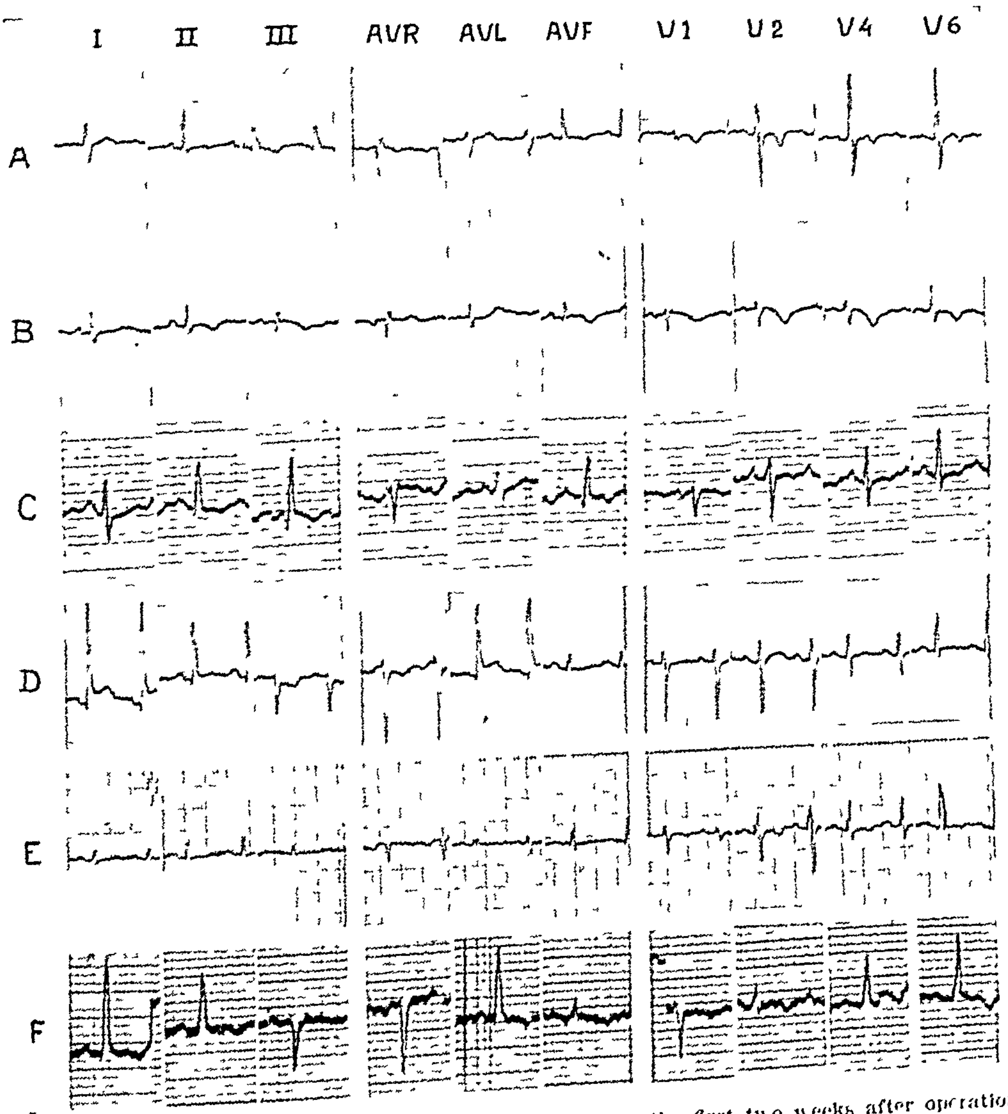

Fig. 1.—Electrocardiograms of 6 patients recorded in the first two weeks after operation.
A, right-sided lobectomy two days after operation.
B, seven days after left-sided pneumonectomy.
C, two days after right-sided lobectomy.
D, two days after left pneumonectomy (note S-T deviation).
E, eleven days after left pneumonectomy (in this case pericardial puncture was necessary).
F, seven days after left pneumonectomy.

In some other patients not all precordial leads were pathologically changed but only part of them, especially the right precordial ones. The T wave in aV$_R$ was isoelectric or negative in nearly all patients. T in aV$_L$ was practically always positive. Every one of these 35 patients showed also abnormalities in the T waves in the standard leads. The T_{III} was always isoelectric or negative; in 5 cases the T_I was abnormal (Fig. 1, *F*).

These S-T and T-wave changes gradually disappeared in most patients, sometimes as early as after a week, in other patients after some weeks or months (Fig. 2). Only in one case did the changes of the T waves persist for longer than six months.

Table III shows the relationship between the occurrence of the S-T and T-wave changes and the nature of the operation. It is a striking feature that the number of cases with abnormalities of the T waves is relatively greater in pneumonectomies than after the other operations. On the other hand, the above-mentioned abnormalities do not arise at all after thoracoplasty.

TABLE III

TYPE OF OPERATION	TOTAL NUMBER OF PATIENTS	S-T AND T-WAVE CHANGES	
		NUMBER	PER CENT
Thoracoplasty	21	0	0
Segmental resection	53	17	32
Lobectomy	19	5	26
Pneumonectomy	20	13	65
Total	113	35	31

We also studied whether there was any relationship between these changes in the T waves and the postoperative arterial oxygen saturation. As no negative T waves were observed after thoracoplasty, we separately considered the thoracoplasty cases in the consecutive series of 28 patients in whom the arterial oxygen saturation was determined during the first postoperative weeks; 8 patients in total.

Table IV contains the data obtained in these 8 patients. It was found that the arterial oxygen saturation can fall to low levels after thoracoplasty. This severe and long-lasting undersaturation was especially observed in the patients in whom thoracoplasty had resulted in a pendular movement of the anterior chest wall, refractory to corrective measures. It must be pointed out that in each instance the lowest oxygen saturations of the first postoperative weeks are listed in the tables. The electrocardiogram and the oxygen saturation were frequently determined, so long as the latter value had not reached

TABLE IV. THORACOPLASTY CASES

CASE NUMBER	OXYGEN SATURATION (%)		S-T- AND T-WAVE CHANGES
	BEFORE OPERATION	AFTER OPERATION	
1	95	94½	Absent
2	96	91	Absent
3	-	93½	Absent
4	92½	90	Absent
5	96	84	Absent
6	96	94	Absent
7	91	89	Absent
8	-	75	Absent

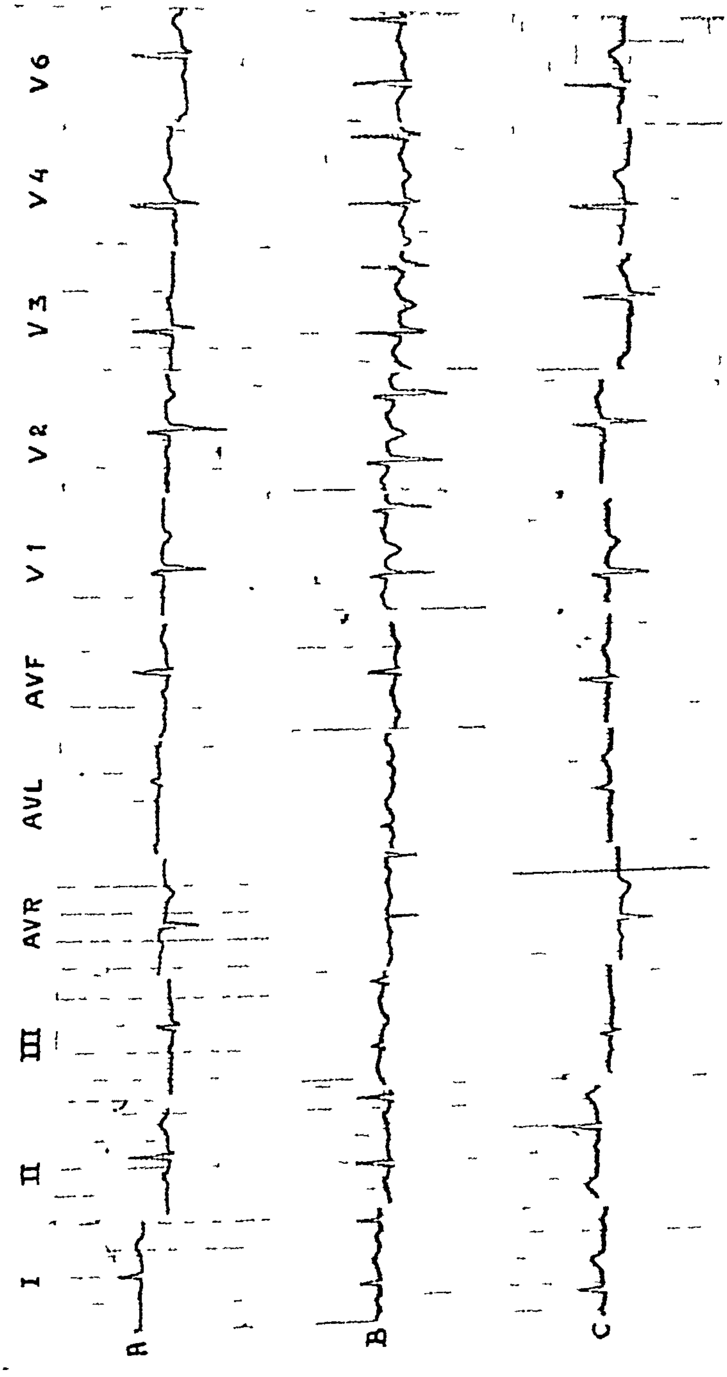

Fig 2.—Electrocardiograms of a patient, 10 years of age *A*, before operation *B*, three days after segmental resection in the right upper lobe *C*, seven months afterward

preoperative levels. This was usually the case in the first postoperative week; Patients 5 and 8 from Table IV, however, needed more than a fortnight before the oxygen saturation had become normal (Maier and Cournand[10]).

It is evident that even a markedly lowered oxygen saturation was not accompanied by electrocardiographic changes as described above. At most, some flattening of the T waves in the first standard lead and in V_6 was observed, probably as an anoxemia reaction.

Table V gives the same data of the 20 other patients, who underwent some form of resection therapy. In these patients also the arterial oxygen saturation sometimes fell considerably in the postoperative period and in a number of them the S-T and T-wave changes arose. On the other hand, these abnormalities were also observed in the patients in whom the oxygen saturation did not fall at all after the operation. The table shows that there does not exist any relationship between the occurrence of the electrocardiographic changes and the arterial oxygen saturation.

TABLE V. RESECTION CASES

CASE NUMBER	OXYGEN SATURATION (%)		S-T- AND T-WAVE CHANGES
	BEFORE OPERATION	AFTER OPERATION	
1	92	79	Absent
2	-	94½	Absent
3	96	90	Absent
4	95	90	Absent
5	95	91	Absent
6	96	95	Absent
7	95	61	Absent
8	95	86	Absent
9	96	93	Absent
10	-	58	Only T_{V_6} negative
11	96	88	Present
12	97	90	Present
13	96	93	Present
14	96	90	Present
15	96	90	Present
16	96	95	Present
17	-	92	Present
18	98	94	Present
19	96	94	Present
20	94½	90	Present

On the basis of the radiologic findings of 63 cases, we finally studied whether there was a correlation between the electrocardiographic abnormalities and the presence of air in the thoracic cavity.

Thirty-two patients had undergone a left-sided resection; in 16 of them the T-wave changes developed in the first week, while only 3 of them had a pneumothorax at the same time. In 6 other patients, however, a pneumothorax was present without any changes on the electrocardiogram. The same ratio was observed in the operations on the right side. It was impossible to demonstrate a relationship between the presence of air in the pleural cavity and the occurrence of electrocardiographic changes.

DISCUSSION

Only in 12 of the 508 patients examined did an abnormal electrocardiogram exist before the operation. This is quite consistent with the investigation carried out by Harden and his associates[6] who found only one abnormal electrocardiogram in 95 patients. Björkman,[4] however, arrived at quite different figures. These differences are probably due to varying opinions on what should be considered normal and abnormal in the electrocardiogram. We must remark that we did not regard an isolated negative T wave in Lead III or aV_F as pathologic. Changes in the position of the heart are generally described after intrathoracic operations (Harden,[6] Weinshell,[15] Hertzman[7]). A distinction must be made between the transient changes of the position of the heart, as observed shortly after the operation, and the final changes found later, for example, after six months. The frequent occurrence of a permanent change in the position after a left pneumonectomy is an especially striking feature.

The change of the position as such may give rise to a flattening or turning negative of the T waves in various leads (Goldberger[18]). An electrocardiogram recorded in the first week following operation may show quite different changes, localized in the T waves and S-T segments. We observed these changes in 35 of the 113 patients examined for this. Negative T waves were found in all precordial leads, while a negative T_I and T_{II} were also observed. In these cases we could speak of massive T-wave changes. These abnormalities were usually of short duration, lasting only for some weeks. T-wave changes have also been described in the literature, but only a few investigators have carried out electrocardiographic investigations during the first postoperative week (for example, Hoppe[8]). It is only possible to get information about these electrocardiographic abnormalities by making an electrocardiogram as early as possible after the operation.

Opinions vary on the cause of the T-wave changes after resection therapy for tuberculosis.

We cannot confirm Weiss's[6] opinion that a displacement of the mediastinum to the left may be an etiological factor. This displacement was observed several times in our patients with left pneumonectomies. The negative T waves usually disappeared rapidly, while the displacement of the mediastinum persisted or even became worse. In the only patient with the persistent negative T waves, the mediastinum was not displaced to the left.

The presence of air between the heart and the chest wall has also been mentioned as a cause of the T-wave changes (Armen[1]). We were unable to find any indications of this.

Lesions of the myocardium ("myocardial damage") due to hypoxia have also been considered responsible (Katz,[9] Rahman[11]). We did not find any correlation between the occurrence of electrocardiographic changes and the arterial undersaturation.

Refrigeration during the operation (Rahman[11]) does not seem to be of importance in this respect.

Digitalis preparations may cause manifest S-T and T-wave changes, which may to some extent simulate the abnormalities described by us. We did not treat our patients with any of these preparations, so that this causative factor can be ruled out.

According to some investigators (Weiss[16]), overloading of the right heart after pulmonary resection might give rise to changes of the electrocardiogram. We did not believe that this is probable, in view of the investigations of Sluiter[13] and Schweitzer[14] who did not observe such T-wave changes even in the case of a completely developed cor pulmonale.

The great similarity to the picture of acute pericarditis was a striking feature in all the cases with extensive T-wave and S-T changes in the postoperative electrocardiogram. Fig. 1, *D* is very illustrative in this respect, as the characteristic S-T elevations are present. This similarity was also observed by the Italian investigator di Maria (cited by Harden[6]). We are, therefore, of the opinion that the electrocardiographic abnormalities found by us, in patients who had undergone pulmonary resection, were attributable to changes of the pericardium. In one of our patients, a pericardial puncture was required, as the circulation had been seriously impeded due to accumulation of fluid in the pericardium (electrocardiogram of this patient: Fig. 1, *E*. This electrocardiogram shows a somewhat later stage in which the S-T elevations are diminished and the changes in the T waves appear). Bérard and Jacquemet[2] observed an increase of fluid in the pericardium in 4 out of 500 cases of pulmonary resection (only after pneumonectomy). They did not carry out electrocardiographic examinations, however.

During the first postoperative period, electrocardiographic changes such as those just described after pulmonary operations are also observed after certain heart operations; commissurotomy of the mitral valve in mitral stenosis, and valvulotomy of the pulmonary valves in cases of pulmonary stenosis. In these cardiac operations, the pericardium is opened and it is conceivable that a condition of irritation of the two pericardial leaves may develop. On several occasions, Campbell and Brock[3] had to perform pericardial puncture after a valvulotomy for pulmonary stenosis. Analogous to this it seems to us that in a number of cases of pulmonary tuberculosis a transient, usually symptomless, pericardial irritation may be caused by intrathoracic operations with manipulations in the neighborhood of the pericardium. This irritation many give rise to a typical electrocardiographic picture, which proves to be reversible in most cases. We were strengthened in this opinion by the absence of electrocardiographic abnormalities in the S-T and T waves after thoracoplasties. Pericardial aspiration would appear to be indicated in cases in which this electrocardiographic picture is observed in combination with a weak pulse and other signs of fluid accumulation in the pericardium.

SUMMARY

1. The electrocardiographic findings in more than 500 patients with pulmonary tuberculosis were studied. Special attention was given to the position of the heart in connection with age. In only 12 patients a pathologic electrocardiogram was found; the abnormalities were mainly located in the T waves.

2. In 444 patients, the electrocardiogram, recorded at least six months after the operation (thoracoplasty and pulmonary resection), was compared with the preoperative electrocardiogram. A change in position of the heart was observed in 94 patients. This change of cardiac position was most frequent after left pneumonectomy.

3. Repeated electrocardiographic examinations were carried out during the first postoperative weeks in 113 consecutive patients from this series. Thirty-five of them showed extensive changes in the S-T and T waves which developed soon after the operation and persisted during a number of weeks but disappeared in most cases within six months after the operation.

4. The cause of these electrocardiographic changes in the postoperative period is discussed. Based on the marked similarity of the electrocardiographic picture with that of pericarditis and on the strength of clinical observations, it is considered probable that the abnormalities found are caused by pericardial irritation.

All the operations mentioned were performed by Prof. L. D. Eerland, M.D. (Director of the Surgical Clinic, University Hospital, Groningen, Holland).

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THE USE OF A PLASTIC SPONGE (IVALON) IN OPERATIVE PROCEDURES FOR PULMONARY TUBERCULOSIS

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THE use of space-taking devices has long been a part of the treatment of pulmonary tuberculosis. It is said that therapeutic injections of air into the thorax were advocated at the time of Hippocrates. Gilchrist,[1] in the middle of the eighteenth century, recommended pneumothorax; and Carson,[2] in 1819, described the pulmonary physiology with an amazing degree of accuracy and advised intrathoracic injections of air for "quiescence" of a diseased lung. Others made similar pleas for some type of collapse therapy of the lung with pneumothorax, but it remained for Forlanini[3] of Italy, in 1882, to describe an aseptic method and specific technique which made pneumothorax a standard of treatment for tuberculosis, and others later to make it a "must" for uncontrollable hemoptysis

DeCerenville,[4] in 1885, described a method of anterior thoracoplasty allowing the cut ends of the ribs to come together and reducing the circumference of the chest to close tuberculous cavities. Others reported similar operations, as did Brauer[5] and Friedrich[6] who popularized massive rib resections in Europe in the early part of the twentieth century. Later, Wilms,[7] Sauerbruch,[8] and Alexander[9] developed the safe, conventional, staged thoracoplasty done today.

Tuffier,[10] in 1891, first described his "extrapleural pneumonolysis" consisting of stripping the pleura from the rib cage over the apex of the lung through an incision in the second interspace anteriorly and letting this space fill with air. In 1901, Sarfert[11] performed a similar procedure, using a gauze packing to maintain the depression of the pleura. Tuffier then reported on similar cases in which he filled the space with grafts of adipose tissue, omentum, and fibromas. Later he used refills of air and paraffin, though the latter filling had been reported first by Baer[12] in 1913. Many other filling substances have been reported: Lilienthal,[13] 1928, gum sheeting; Alexander,[14] 1930, autogenous grafts of pectoral muscles; Oughterson and Harvey,[15] rubber bags; Eloesser,[16] 1932, paraffin.

With the development of pulmonary resections in the treatment of pulmonary tuberculosis, there was an even greater stimulus for further search for space-taking devices. Since the introduction of plastics, various of these materials have been used for the purpose of filling spaces in the bony thorax in the treatment of tuberculosis. Lucite balls were first used by Wilson and Baker.[17] Later, the use of Polystan (polyethylene) sponges were reported by

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Bing and associates.[18] In 1951, Grindlay and Waugh[19] at Mayo Clinic reported the use of a formalinized polyvinyl alcohol sponge in various parts of the body, including the chest.

Conventional thoracoplasties, while having the advantage of being entirely nonirritating, have the marked disadvantages of requiring several stages and leaving large portions of chest flail, making for respiratory difficulties during the healing period. Unfortunately, in addition, they impair the breathing capacities, as well as being extremely disfiguring. Plombage, for either primary or secondary procedures, has the advantages of being a single procedure, of avoiding the flail chest, reducing the operative risk, obtaining better and more exact collapse, and reducing pain and loss of mobility of the adjacent arm. Equally as important is the preservation of pulmonary function. Watson and Gaensler[20] demonstrated a drop in pulmonary function of only about one-half as much with plombage thoracoplasty as with conventional

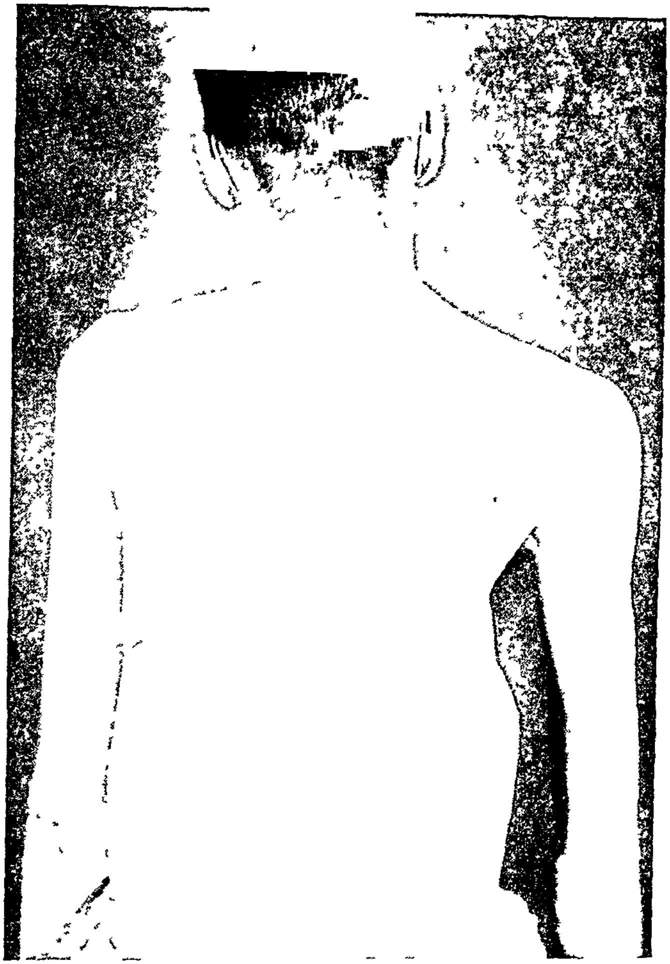

Fig. 1.—Photograph of M. H., 28-year-old white man, who had a conventional three-stage, 7-rib thoracoplasty on the right, a 7-rib plombage thoracoplasty on the left. Note the scoliosis, rib cage deformity, elevated shoulder and muscular atrophy on the right secondary to the conventional thoracoplasty.

thoracoplasty, this work being done on patients who had Lucite balls used for the plombage thoracoplasty. This makes it feasible to operate unilaterally and bilaterally on poor-risk patients that could not tolerate the classic staged thoracoplasty (Fig. 1).

Of further importance is a factor that has not been sufficiently stressed. Economically, the plombage procedures offer a tremendous saving in many ways, especially in the presence of a backlog of patients. In a given time, with a fixed personnel, more plombage procedures can be performed than conventional thoracoplasties or resections. Postoperatively, the patients do extremely well and require far less care. In addition, a shorter period of total and postoperative hospitalization is required for these patients.

MATERIAL USED AS PLOMBAGE

As just mentioned, many different types of material have been used for plombage and reported upon. Each has its advantages and disadvantages. Growing body tissues would seem to be the most desirable. However, the obvious disadvantages are that they are difficult to obtain, scant in quantity, and tend to atrophy. Inert foreign substances would, of course, seem to be the next most feasible. Ideally they should be: (1) stable, (2) chemically inactive, (3) compressible, (4) able to produce minimal foreign body response, (5) easily shaped, (6) light in weight, and (7) of such a nature that they might be incorporated into the body tissues. That such properties may be assigned to formalinized polyvinyl alcohol sponges was first demonstrated by Grindlay and Waugh,[19] and later confirmed by Gale and co-workers.[21] We have used this type sponge exclusively.

TYPES OF OPERATIONS AND INDICATIONS

At the Mississippi State Sanatorium, we have been using the Ivalon sponge for a period of four years. This report deals with the utilization of 150 sponges in the first 144 consecutive patients who have been followed from one to four years.

The plombage procedures are classified as "Primary" if used for collapse and as "Secondary" if used as space fillers following resection. The primary procedures were usually plombage thoracoplasties in which the sponge was placed subcostally, extraperiosteally as a permanent implant. In some cases, however, the sponge was placed extrapleurally after stripping the parietal pleura from the chest wall.

Secondary plombages were usually placed extrapleurally or extraperiosteally as a tailoring procedure in cases where a lobe or more pulmonary tissue was removed and the remaining lung did not readily fill the hemithorax. These plombages were preferably introduced as a part of the same operation wherein the resection was carried out, though some were done at a second operation. In a few cases, following pneumonectomies, the sponges were introduced intrapleurally after a minimum postoperative delay of three months.

Indications for the secondary space-filling procedures are essentially as outlined by Cotton and Paulsen[22] and may be briefly summarized as the prevention of overdistention if (1) the remaining lung will *not easily fill* the chest or if (2) extensive residual disease, particularly of the contralateral apex, makes any degree of overdistention undesirable. The indications for the primary plombage are somewhat more controversial even though, basically, they were set forth by Tuffier,[23] the originator of "extrapleural pneumonolysis" and have changed very little since that time. These are: (1) uncontrollable hemoptysis from an apical lesion, (2) fibrocavitary tuberculosis limited to the upper portion of the lung, (3) supple cavities less than 4 cm. in size, (4) lesions of tuberculosis which might well be resected but for a positive sputum with bacilli resistant to present antituberculous medications. In addition, (5) plombage thoracoplasties are preferred for unilateral and bilateral procedures in patients with markedly impaired respiratory reserves, and (6) where the distribution of the disease makes resection of all *significant* residuals impractical or impossible, but allows collapse of open cavitary apical lesions.

The primary extrapleural plombage has been used only in cases where there is some good evidence of healing of the disease prior to surgery and there is no evidence of cavitation within a centimeter of the periphery of the lung. With extreme care in the selection of patients, avoiding the pitfalls emphasized by Zimmerman and his associates,[24] the risks should be minimal, but at the same time the scope of this procedure will be severely limited.

OPERATIVE METHODS

Primary plombage thoracoplasties are performed much as originally outlined by Wilson and Baker.[17] The usual parascapular incision is made as for a conventional thoracoplasty and the inferior extent of the thoracoplasty is determined by the usual criteria for conventional thoracoplasty. Beginning with the lowest rib and continuing through the first, the periosteum is stripped only from the under surface of the rib, leaving the outer surface intact. The posterior muscle bundles are dissected free from the ribs subperiosteally by blunt dissection around to the vertebral column. The intercostal muscles are divided posteriorly after being separated from the associated intercostal vessels and nerves which are carefully preserved.

The subcostal space is irrigated, hemostasis achieved as carefully as possible, and an Ivalon sponge is tailored to fit. Extra sponge is added anteriorly or laterally between the original sponge and the rib cage to achieve the desired compression. Penicillin and streptomycin, in relatively large quantities, are injected into the sponge and the sponges are sutured in place to each other and/or to adjacent ribs. A single drainage tube is inserted into the subcostal space through an inferior stab wound in the midaxillary line and attached to a water-seal drainage system. If the pleura has been opened accidentally, a thoracotomy tube is inserted intrapleurally and connected with a drainage bottle. The wounds are closed in layers, and a pressure dressing and a snug-fitting chest binder are applied. Drainage tubes are removed when they cease to drain (usually about the third postoperative day). It is quite routine to

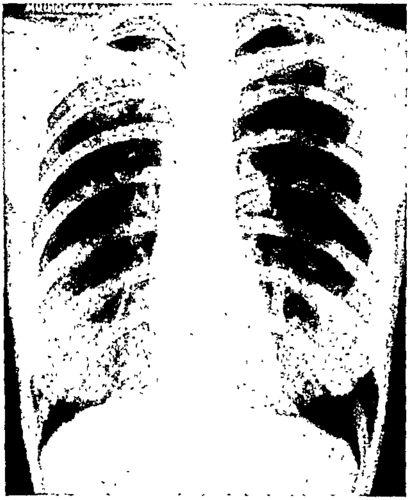

Fig. 2*A*.— Preoperative roentgenogram of M. J. J., a 21-year-old white woman with residual apical fibrocavitary disease.

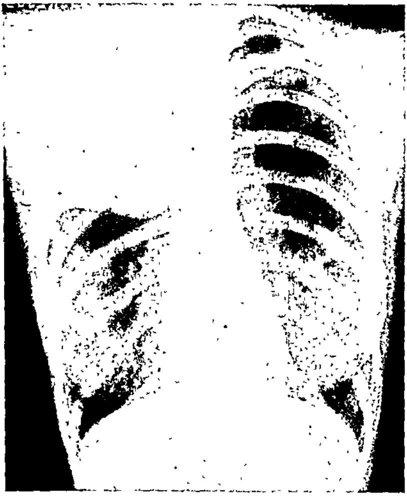

Fig. 2*B*.—Postoperative roentgenogram after 6-rib plombage thoracoplasty.

collect 800 to 1,000 c.c. of serosanguineous drainage from the wound. If this is not drained, more collapse of the lung is obtained than is desired, and this extra fluid may add to the dangers of infection (Figs. 2 and 3).

The method of introducing the primary extrapleural plombage varies in that a short posterior incision is made and a 10 cm. length of the fourth rib is resected. The endothoracic fascial plane is entered and the parietal pleura freed by blunt dissection over the entire area which is to be collapsed. The opened space is then filled with the shaped sponge which may be sutured to the chest wall. The usual penicillin and streptomycin are introduced into the sponge and the area drained as in the extraperiosteal plombage. Postoperative treatment is the same (Figs. 4*A* and 4*B*).

Insertion of the plombage in the extrapleural space following resection is usually done at the time of resection. Following completion of the lobectomy, the pleura is separated from the chest wall beginning at the site of the thoracotomy and extending over the same area as was filled by the resected lung. A sponge is cut to fit the extrapleural space and sutured in place. After a drainage tube has been inserted anterolaterally, the edge of the pleura is sutured back to the chest wall, extrapleuralizing the entire sponge. In those cases where the pleura has been torn or where a partial pleurectomy was required in the original resection, the pleura is repaired in order to be certain that the sponge is walled off from the remaining lung tissue. Only occasionally has there been insufficient pleura to cover the sponge. In these cases, it is usually preferable to do a plombage thoracoplasty or to insert the extrapleural sponge at a later date (Figs. 5*A* and 5*B*).

Most of the plombes used with resection have been with upper lobectomies. However, three sponges have been used following lower lobectomy. One of these was placed in the apex above a depressed right upper lobe after bilobectomy, and two were placed in the extrapleural space formed by dissecting the lower posterolateral pleura free as far as the edge of the diaphragm and then inserting the sponge in this pocket. The sponge extended over the posterior half of the diaphragm, thus leaving the diaphragm intact and functioning over its anterior half. No ill effects have been noted.

In cases where it is desirable to achieve simultaneous collapse with a resection, but most of the parietal pleura has been stripped off or there has been contamination of the pleural space, a plombage thoracoplasty is performed. This secondary thoracoplasty is carried out in a manner similar to the primary plombage thoracoplasty, except that an attempt is made here to follow the limits of the removed lung tissue rather than square off the lower margin as is done in primary thoracoplasties.

Postresection plombages as second procedures have been carried out for the same indications as those above except in situations where the filling ability of the lung was misjudged, where the original procedure had been of considerable duration, or where there was considerable contamination. As a general rule, this is an unsatisfactory procedure since it is difficult to follow the outlines of the resected lung tissue at a second operation and it probably collapses considerable usable lung tissue.

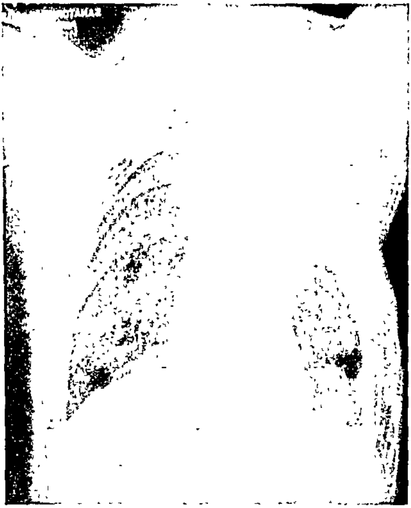

Fig. 3A.—Preoperative roentgenograms of C. W., a 44-year-old white man, who had had a left three-stage 8-rib thoracoplasty five years previously. Patient has a marked kyphoscoliosis and a maximum breathing capacity reduced to 60 L. per minute.

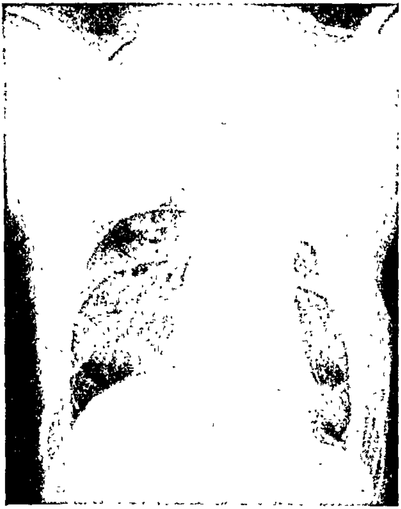

Fig. 3B.—Roentgenogram after right 7-rib plombage thoracoplasty was performed for the fibrocavitary residual with conversion of the sputum and no change in the maximum breathing capacity.

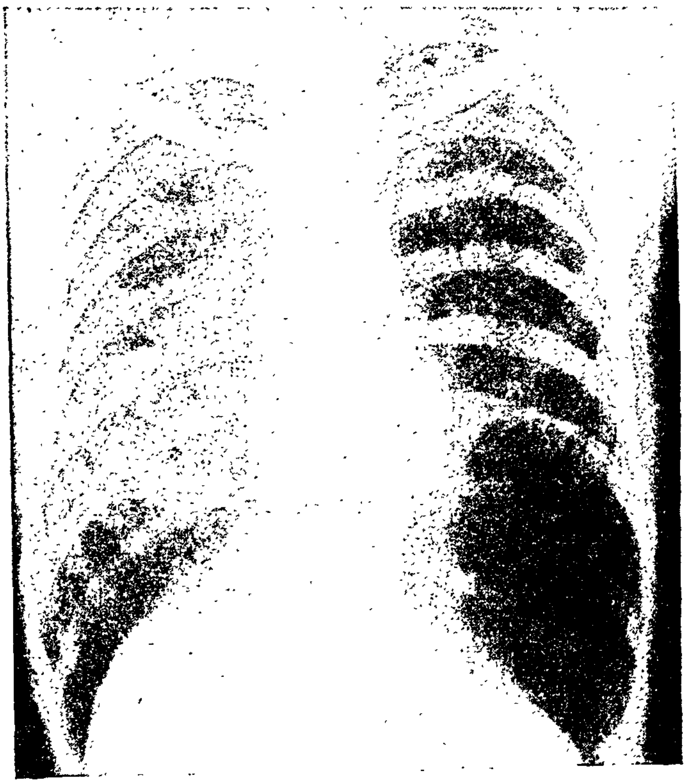

Fig. 4A.—Preoperative roentgenogram of C. G., a 40-year-old white man with bilateral apical fibrocavitary disease.

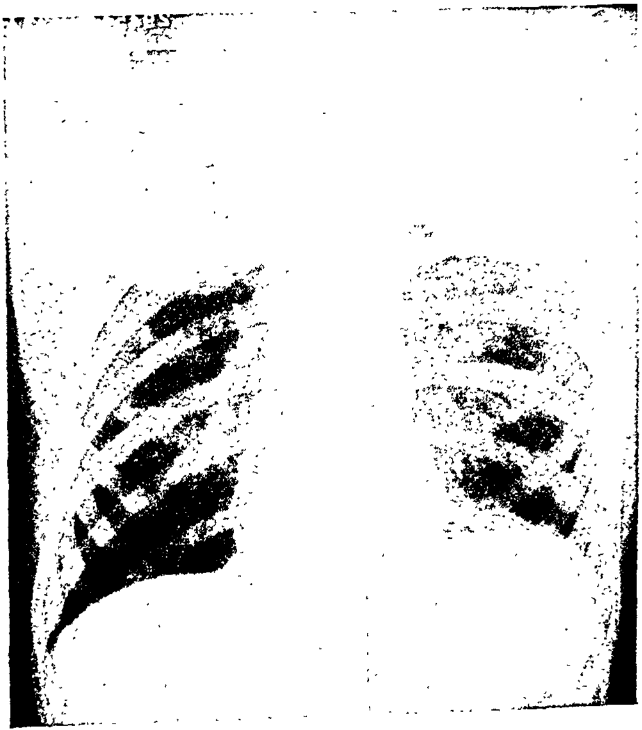

Fig 4B.—Roentgenogram after right extrapleural plombage and left plombage thoracoplasty. The sputum converted and there is no clinical or laboratory evidence of respiratory embarrassment.

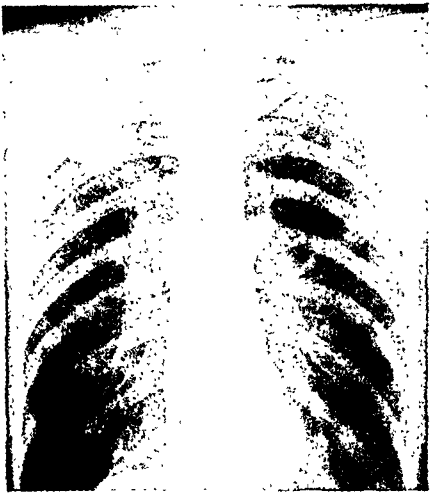

Fig. 5A.—Preoperative roentgenogram of J. W. A., a 54-year-old white man, with an undiagnosed lesion in the right upper lung.

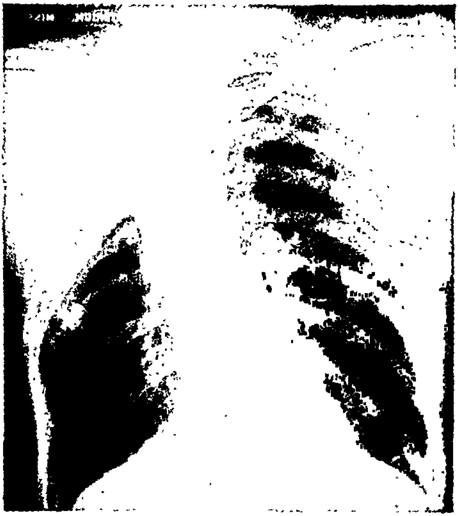

Fig. 5B.—Postoperative roentgenogram after resection of the right upper and right middle lobes for a tuberculous granuloma with a concomitant 5-rib plombage thoracoplasty. The patient continues to do well three years later.

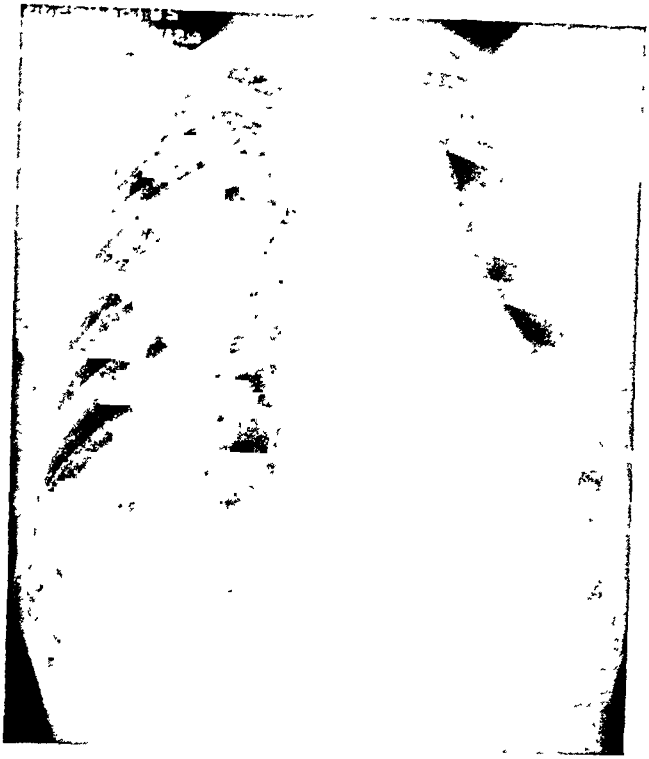

Fig. 6*A*.—Roentgenogram of W. K. C., a 58-year-old white man, following left pneumonectomy for destroyed lung with sputum positive with resistant bacilli. Note mediastinal shift and marked herniation of lung through anterior mediastinum. Patient had right heart failure in early postoperative period.

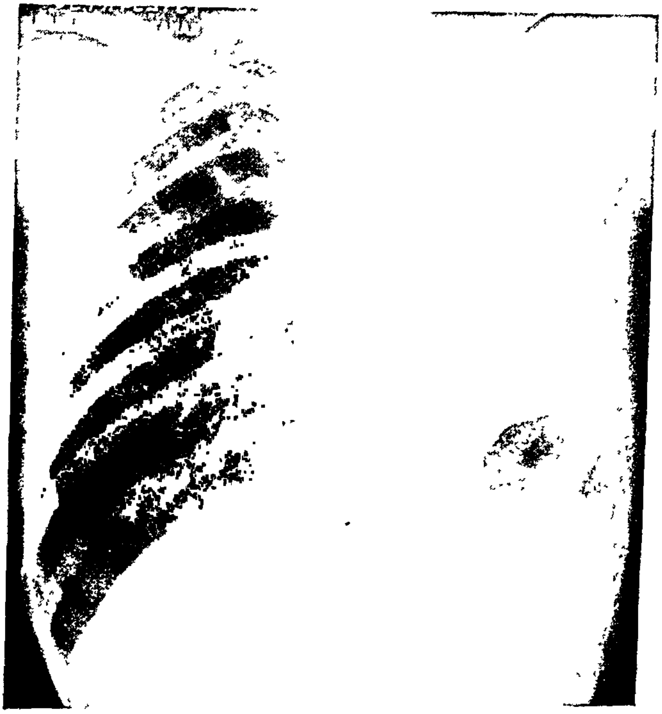

Fig. 6*B*.—Roentgenogram after insertion of Ivalon sponge into left pleural space three months postpneumonectomy. Patient became much less dyspneic, though symptoms of cor pulmonale have persisted intermittently during the subsequent two years. Sputum remains negative.

The last method of plombage concerns its intrapleural use following pneumonectomies. Most of these pneumonectomies have been for destroyed lungs; and the parietal pleura, or a large portion thereof, has been removed with the diseased lung. Since it is felt that in many cases the field may be contaminated, no sponge has been inserted intrapleurally at the time of the primary operation but is inserted at a second procedure at least three months later. As the majority of bronchopleural fistulas or empyemas will become evident within this time, it seems safer to wait this period. Gale and associates[21] have warned about the increased dangers of infection if a sponge is placed intrapleurally at the time of operation.

Since the sixth rib has usually been resected for the pneumonectomy, the chest is re-entered through an interspace, as resection of the fifth rib creates sufficient deformity for the scapula to impinge beneath the seventh rib. At the time of the second operation, the coagulated fluid is removed and examination of the space demonstrates a thick fibrous peel over the entire chest cavity, almost invariably drawing the mediastinum toward the operated side for a distance of 2 to 5 cm. The fibrous peel is separated at its mediastinal edges, thus allowing the mediastinum to return to the midline. A sponge is then cut to fit the space snugly (but not tightly); penicillin and streptomycin are added, and the chest wall is closed in layers with drainage (Figs. 6*A* and 6*B*).

RESULTS

One hundred and fifty plombage procedures had been performed from one to four years prior to the completion of this study. Seventy-five have been primary plombage thoracoplasties and 10 have been primary extrapleural plombages. There have been 31 extrapleural plombages performed as a single procedure with a resection, and 12 plombage thoracoplasties simultaneous with resection. Of these latter, 4 were associated with pneumonectomies while the others were with lobectomies or their equivalent. There has been only one postresection extrapleural plombage performed as a second operation. Of 9 postresection plombage thoracoplasties, done as second-stage operations, 5 followed pneumonectomies, and 4 followed lobectomies. Twelve intrapleural plombages have been used, all following pneumonectomies.

TABLE I. IVALON SPONGES USED AT M.S.S.
July 1, 1952—June 30, 1955

Plombage thoracoplasty, primary	75
Extrapleural plombage, primary	10
Concomitant lobectomy and extrapleural plombage	31
Concomitant lobectomy and plombage thoracoplasty	8
Concomitant pneumonectomy and plombage thoracoplasty	4
Postlobectomy extrapleural plombage	1
Postlobectomy plombage thoracoplasty	4
Postpneumonectomy plombage thoracoplasty	5
Postpneumonectomy intrapleural plombage	12
Total	150

Infection has been the chief concern. However, to date, only 3 sponges have had to be removed for infection. Two had been used for plombage thoracoplasties and started draining five and twenty-four months, respectively, postoperatively. The sponges were easily removed and the ribs resected in a conversion thoracoplasty with early healing. One extrapleural sponge inserted at the time of a left upper lobectomy became infected and the patient developed a bronchopleural fistula three months postoperatively. The sponge was removed and the space drained, with spontaneous closure of the bronchopleural fistula the following day. A limited thoracoplasty subsequently has given complete healing. In no case has there been a tuberculous infection.

We have been willing to accept a small percentage of infections, feeling that the advantages outweigh this disadvantage. The chest becomes stabilized in two or three months, and it is a simple matter to remove the sponge and convert to a conventional thoracoplasty should infection intervene.

Desforges, Gibbons, and Strieder[25] have reported finding positive cultures for acid-fast bacilli in the subcostal fluid in 12 of 32 cases of plombage thoracoplasty, *done essentially by the same technique as just described, but using* Lucite balls. As the tubercle bacilli must certainly be present in the region of the sponge in many of our cases, it has been surprising that none have developed a clinical tuberculous infection. Perhaps the fact that the Ivalon sponge is incorporated into the body tissues, and does not create a permanent nidus of fluid, may in part account for this.

There has been one death associated with these procedures. In this case, cardiac arrest occurred just as the skin had been closed following insertion of a sponge intrapleurally three months after a right pneumonectomy. The incision was immediately reopened, but some extra time was required in removing the sponge before cardiac massage could be started. A full physical recovery was obtained, but there was marked cerebral damage. At the end of one month, during which gradual mental improvement had been noted, sudden death occurred due to a pulmonary embolus, proved by autopsy.

We have had one other complication, this being in a patient who had a pneumonectomy and subsequent 9-rib plombage thoracoplasty. Six weeks following the thoracoplasty, the patient began to complain of numbness, tingling and, later, weakness in the ulnar side of her arm, leading to her later hospitalization at an outside hospital. Subsequently, operation demonstrated a dense layer of fibrous tissue surrounding the third trunk of the brachial plexus which was freed with gradual subsidence of symptoms.[26]

Of some concern to us at the beginning of this series was the fate of the ribs stripped of their periosteum and, indeed, the first thoracoplasties were carried out with complete stripping of the ribs. Many of these ribs atrophied to the extent that they broke spontaneously. However, ribs that have been stripped of periosteum only from the under surface do not atrophy and appear quite normal both histologically (Fig. 7) and roentgenographically.

Some of the sponges have been re-examined as long as twenty-six months following their insertion. If infected, the sponge lay in a sea of purulent fluid and could be easily lifted out. Usually, however, the sponges were firmly fixed by an ingrowth of fibrous tissue for 5 to 10 mm., appeared grossly quite normal and retained the original resiliency. They were moist, but not accompanied by overt fluid accumulations. Some mild pleuritic inflammation appeared to have been incited, as there was always a moderately adherent symphysis of the visceral and parietal pleurae underlying an extrapleural sponge.

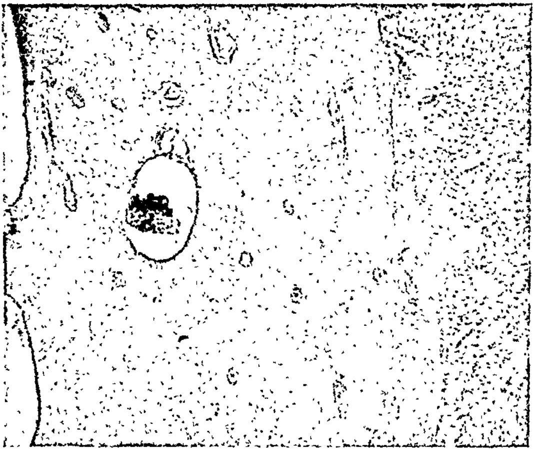

Fig. 7.—Photomicrograph (×200) of rib that had been completely deperiostealized twenty-six months previously at postpneumonectomy plombage thoracoplasty (L. B. B.). The bone appears viable with normal marrow elements. There is dense fibrous tissue surrounding the rib instead of periosteum. Grossly, this rib had atrophied to about one fourth of its previous diameter.

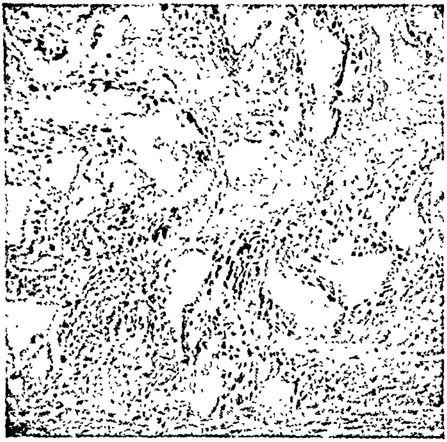

Fig. 8.—Photomicrograph (×200) of Ivalon sponge (light areas) enmeshed with fibrous connective tissue of moderate vascularity. Foreign body giant cells are present.

Microscopically, the ingrowth of fibrous connective tissue was demonstrated with varying numbers of foreign body giant cells and collections of hemosiderin pigment (Fig. 8). The infected sponges showed a tremendous increase in pigmentation and foreign body reaction.

The therapeutic results of the primary plombage thoracoplasties and primary extrapleural plombages as definitive procedures for tuberculosis have been gratifying. Of the 80 patients with 85 primary plombage procedures followed from one to four years, 72 have had their disease controlled by the collapse alone. One continues to have a positive sputum intermittently, but is deemed unsuitable for any further procedure. The other 7 have required additional surgery and a full account of this experience will be the subject of a subsequent report.

Recently it has been demonstrated that virtually any plastic polymer implanted into the subcutaneous tissues of rats will act as a carcinogenic agent, usually producing a fibrosarcoma.[27, 28] These various ploymers included Cellophane, Dacron, polyethylene, polyvinylchloride, Silastic, Pliofilm, nylon, polymethyl methacrylate, polystyrene, Saran, Ivalon, Kel-F, Teflon, and even silk. These agents required roughly fourteen to twenty-eight months to produce tumors, thus usually taking over one-half the life span in a susceptible strain of rat. Whether this can be applied to human experience cannot be readily determined, but it would seem wise, perhaps, to restrict the use of any implanted plastic material in clinical use to the older age groups.

SUMMARY AND CONCLUSION

The history of the development of the thoracoplasty and other space-filling devices in the treatment of tuberculosis is briefly reviewed. Experience with the use of 150 Ivalon (formalinized polyvinyl alcohol) sponges, used as space-taking devices in 144 patients over a period of three years at the Mississippi State Sanatorium, is reported. Of these 150 sponges, 75 were primary plombage thoracoplasties, 10 primary extrapleural plombages, and the remainder postresection plombages used either concomitantly with the resection or as a second procedure.

Of these sponges, 3 have become infected. Following their removal and conversion thoracoplasty, healing has occurred in all. No tuberculous infection has been encountered. One other sponge was removed because of fibrosis involving the third trunk of the brachial plexus.

Plombage has proved itself a desirable operation in the treatment of pulmonary tuberculosis, both for primary collapse therapy and as a space-filling device following resections. While the final evaluation of a foreign substance cannot be determined by four years' observation, the Ivalon sponge appears to be a satisfactory material with an acceptably low incidence of complications.

ADDENDUM

There have been no further complications due to the implanted sponges to date. (Feb. 20, 1957.)

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PLOMBAGE IN THE SURGICAL TREATMENT OF PULMONARY TUBERCULOSIS

A Study of 400 Cases

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THE purpose of this work is to show the advantages and disadvantages of the use of methylmethacrylate (Lucite) balls in the surgical treatment of pulmonary tuberculosis, with special consideration of the long-term tolerance to such a prosthesis. This will be an opportunity to state our position as to the present indications for surgical collapse therapy vs. lung resection.

We present the results of the first 400 operations in which one of us (H. J.) used a prosthesis. There were 345 lung collapses and 55 resections performed between January, 1950, and May, 1955. We excluded from the study the cases in which the balls were deliberately removed within a few weeks after the operation, for example, when they were inserted during a first-stage thoracoplasty and removed at the second stage.

We used hollow spheres, 2.8 cm. in diameter, made of pure methylmethacrylate without the addition of any other plastic material. They can be sterilized in boiling water without alteration in shape.

A spongy material, Ivalon, did not prove satisfactory. The sponges absorbed bloody fluid, their weight increased up to ten times, and fever and pain were observed during the postoperative course.

A material molded and coated with Polystan was discarded because of its heavy weight and its stiffness, which made it hard to position and dangerous to neighboring structures.

SURGICAL COLLAPSE WITH PLOMBAGE

Our 345 cases of collapse with permanent prosthesis include 107 one-stage thoracoplasties, 75 multistaged thoracoplastics, 157 extramusculoperiosteal pneumolyses ("bird-cage" of Lucas and Cleland[6]), and 6 cases of revisional surgery. Table I compares these different types of collapse therapy with permanent plombage over a period of six years.

1. *Technical Details.*—In one-stage thoracoplasties, some ribs are resected, others are simply ruginated. One tries to obtain a satisfactory collapse with as economical a

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TABLE I. DISTRIBUTION PER YEAR OF THORACOPLASTIES AND EXTRAMUSCULOPERIOSTEAL PNEUMOLYSES, WITH AND WITHOUT PERMANENT PLOMBAGE

OPERATIONS	YEARS					
	1950	1951	1952	1953	1954	1955
One-stage thoracoplasties with permanent plombage	1	13	37	32	25	6
Multistage thoracoplasties with permanent plombage	7	15	41	11	–	–
Extramusculoperiosteal pneumolyses with plombage	3	30	23	29	48	48
Revisional collapses with permanent plombage	3	3	–	–	–	–
One-stage thoracoplasties without permanent plombage	9	8	7	5	4	11
Multistage thoracoplasties without permanent plombage	67	29	15	1	1	1
Total operations: 533 Totals	90	98	123	78	78	66

rib resection as possible. In most of our cases, the posterior arc of the fourth rib, the posterior and lateral aspects of the third rib, and the greater part of the second rib were resected, plus a costovertebral disarticulation. Then the lateral border and the inferior face of the first rib were ruginated. In some cases, the posterior arc of the fourth rib was ruginated but not resected. The intercostal muscles were cut at the level of the lateral border of the paravertebral muscles but were not severed from the lateral aspect of the pleura and their vessels were carefully preserved to maintain a good blood supply to the muscles and to the pleura. One may lower the apex slightly by blunt dissection with a small sponge on a clamp but should avoid a too extensive liberation of the lung on its mediastinal aspect. Thus, the space for the prosthesis is limited above by the inferior face of the first rib which separates it from the vessels and nerves of the base of the neck, and medially and below by the intercostal muscles and periosteal beds which protect the mediastinum and the lung. The space is loosely filled with Lucite balls, varying from eight to twelve in number, according to the volume of the space. Such a thoracoplasty collapses the upper third of the lung. We use it for small apical lesions with cavitation.

Multistage thoracoplasties with permanent plombage were used from 1950 to 1953, inclusive, following which extramusculoperiosteal collapse was preferred, because thoracoplasties of six ribs or more required several operations, involved the base of the lung, and impaired respiratory function.

Extramusculoperiosteal pneumolyses have always been followed by plombage. Ribs were neither cut nor removed, but ruginated, usually five to seven of them, the liberation being pushed far forward at the level of the upper ribs. Care was taken, as in thoracoplasties with plombage, to preserve a continuous wall of intercostal muscles and periosteal beds protecting the mediastinum and the lung. The lateral wall of the space is rigid, being composed of the bare ribs. The volume of the plombage should be such that it would collapse the diseased lung completely and prevent any re-expansion of the lung along the mediastinum. It should exert no pressure upon the mediastinal vessels. The usual number of balls inserted is from twenty to thirty. Such an operation collapses at least one half of the lung and does not interfere with the function of the base. It is used in the treatment of cavitary lesions requiring an extensive collapse.

regained the pretreatment leukocyte level within 20 minutes. Although differential leukocyte counts were not obtained in all animals at the 30-minute period after replacement, it was evident that this rapid replenishment of blood leukocytes in the normal rat was accomplished by both granulocytes and lymphocytes, so that the absolute levels of the 2 cell types had returned to the base-line level. Even at the end of the observation period there was little change in the leukocyte pattern.

In the animals that were acutely splenectomized (splenectomy within 10 minutes before the start of the replacement transfusion) the results were similar to those of the normal animals. However, the recovery of the leukocyte count was more delayed and the granulocyte level was increased at the end of the observation period. The sham-operated control group for the acute splenectomy series showed prompt return of the white cell count to above base-line levels.

The chronic splenectomized group (splenectomy 3 to 5 days before study) had a marked leukocytosis at the beginning of the study, consisting of absolute increases in both granulocytes and lymphocytes. In these animals replacement transfusion effected a marked decrease in leukocyte level, with return of the count only to normal levels during the observation period. The cells returning to the circulation consisted of both granulocytes and lymphocytes in a proportion approximating their initial ratios.

The leukocyte count of the leukemic rats also fell precipitously during replacement transfusion. There was an immediate return of cells into the circulation (22,000/c.mm. within 10 minutes), but later the increase was more gradual. The original leukocyte level was much higher than that obtained at the end of the 90-minute observation time following leukocyte removal.

B. *Intermittent (20 Ml.) Replacement Transfusions.*—The same experimental groups of animals (except for the sham-operated controls) were studied during 3 20 ml. replacement transfusions 30 minutes apart. This volume of blood represents about 80 per cent blood replacement. The mean leukocyte responses are plotted in Fig. 3. The replacement transfusions (depicted by the dotted areas) caused a prompt fall in leukocyte count of the normal animals each time, but during the 30-minute recovery period the counts returned to near base-line levels. During recovery, both granulocytes and lymphocytes were returned to the circulation in normal numbers. The mean counts in the last point on the chart are derived from only 3 of the 4 animals in the group, but it seems evident that only moderate granulocytosis occurred at the end of the experiment. 90 minutes after the last replacement transfusion.

The acutely splenectomized animals again exhibited less rapid recovery of the arterial leukocyte levels after replacement transfusion. Each replacement transfusion, therefore, depressed the white cell count progressively. The experiments failed to demonstrate a predominance of either lymphoid or granulocytic elements replenishing the circulation.

The animals that had been splenectomized for 3 to 5 days recovered only to a minimal extent after the replacement transfusions and their initial leukocytosis was decreased in stepwise fashion by the 3 replacement transfusions. As in the previous studies, although postsplenectomy granulocytosis was evident in the initial counts, both cell types participated in the leukocyte recovery periods.

Of the 6 leukemic animals studied, 4 had leukocyte counts below 100,000 per cubic millimeter, and 2 had very high leukocyte counts. The mean values of all 6 animals, as plotted in Fig. 3, reveal a sharp decrease in count with each replacement and poor recovery between procedures. It is of interest that the 2 leukemic animals with the very high leukocyte counts exhibited greater percentage fall with replacement and poorer percentage recovery than the animals with the lower total leukocyte counts. These 2 rats had counts *of 453,000 and 262,500 per cubic millimeter at the beginning of the experiment,* and fell to 44,500 and 27,500 per cubic millimeter, respectively, after the last replacement, with very little change during the final 90 minute observation period.

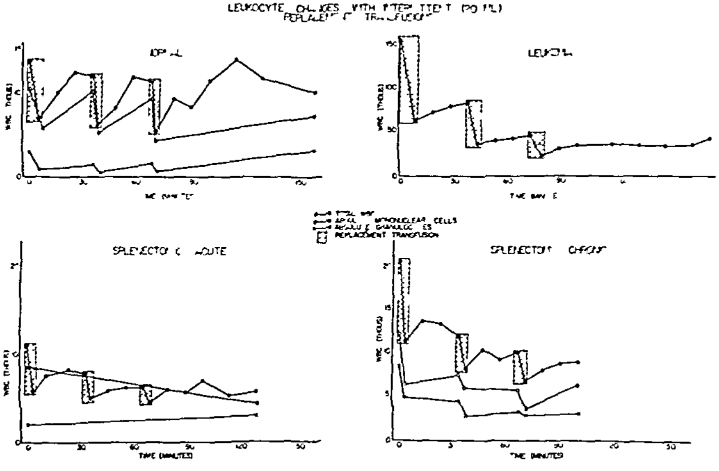

Fig 3.—The mean total and differential leukocyte counts of 4 groups of rats during and after intermittent leukocyte replacement transfusions are shown above. Each stippled column represents the period during which a 20 ml. replacement transfusion was carried out.

In all types of experiments leukocyte counts were performed on the pooled blood obtained from the animals during replacement transfusion. The count of this blood was always approximately the mean of the count before and after replacement. It was evident, therefore, that reductions in leukocyte counts were entirely due to leukocyte removal, and did not represent sequestration of leukocytes within the animal.

from the bone marrow. However, the bone marrow is rich in capillary network and this network may serve as a reservoir of varying importance in different animal species. The leukopheresis procedure of Craddock induced a lymphopenia presumably due to stress of the procedure rather than to lymphocyte removal, so that his studies actually were concerned only with granulocytic replacement. The marked leukopenia persisting for 2 to 3 hours that was induced by leukopheresis may represent either species difference or differences due to the techniques employed. Certainly, the studies of the dog yield little data on the readily available pool of lymphocytes, yet Osgood's observations[3] suggest that the store of mature lymphocytes in man is vast.

SUMMARY

1. Leukocyte removal was accomplished in rats by replacement transfusion of normal, splenectomized, and leukemic animals by normal rat blood rendered leukopenic by mechanical removal of leukocytes.

2. The normal rat mobilized leukocytes rapidly, so that severe leukopenia did not develop during the period of replacement transfusion and recovery to normal leukocyte levels occurred within 30 minutes.

3. Splenectomized rats, whether splenectomy was performed immediately before or 3 to 5 days before replacement transfusion, did not mobilize leukocytes as rapidly as the normal animal. It was evident that these animals had available leukocyte stores, however, since severe leukopenia was not induced.

4. Rats with Shay granulocytic chloroleukemia also had available leukocyte reserves, but marked decrease in leukocyte count was obtained by replacement transfusion. It was evident that the available leukocyte reserves in these animals were not increased in proportion to the circulating leukocyte level.

5. In all experiments the leukocytes mobilized as a result of sudden leukocyte removal were mature and of the same cell type as in the circulation of the animals. This suggested that the leukocyte reserves of the rat consist of a premixed population of granulocytes and lymphocytes, rather than individual granulocytic and lymphocytic storage depots.

6. It was suggested that the immediately available leukocyte reserves of the rat consist of both granulocytes and lymphocytes stored within vascular channels and that the splenic vascular bed constitutes only one portion of that leukocyte "pool."

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A STUDY OF THE PLASMA THROMBIN TIME

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INTRODUCTION

THE conversion of fibrinogen to fibrin constitutes the third or final stage of blood coagulation This conversion may be accomplished by the addition of thrombin to plasma in vitro The time required for the formation of a plasma fibrin clot upon the addition of thrombin is defined as the plasma thrombin time. Prolongation of the plasma thrombin time has been reported in many diseases, including cirrhosis, metastatic carcinoma to the liver, multiple myeloma, severe pre-eclampsia, eclampsia, amniotic fluid embolism, disseminated lupus erythematosus, malabsorption syndrome, Waldenström's macroglobulinemia, and in the newborn.[1-10] However, the basic mechanisms underlying the prolonged plasma thrombin time are not well understood. The conversion of purified preparations of fibrinogen to fibrin by thrombin is influenced by the temperature, pH, calcium concentration, and colloidal state of the substrate.[11] Platelet extracts (platelet factor 2) have been shown to accelerate the interaction of purified preparations of fibrinogen and thrombin.[12] The thrombin time of normal heparinized plasma increases upon storage, presumably due to increased heparinoid activity[13] and may be shortened by the addition of serum or plasma pseudoglobulin.[14] Increase in plasma antithrombin may be a contributing factor. Pronounced plasma hypofibrinogenemia appears to be an infrequent cause for a delayed thrombin time. The abnormal plasma proteins in multiple myeloma may occasionally interfere with or inhibit the conversion of fibrinogen to fibrin.[1, 2, 5, 7] Fibrinogen itself may be qualitatively abnormal and less reactive to the clotting action of thrombin.[8-10, 15-17] Deficiency of a plasma factor which accelerates the conversion of fibrinogen to fibrin has been recently postulated.[6]

Because the mechanisms responsible for prolongation of the plasma thrombin time are not completely understood, a study of the plasma thrombin time was carried out in a variety of diseases. The prolonged plasma thrombin times in this study were found to be due to a deficiency of plasma "albumin," elevation of the plasma fibrinogen level, and, in rare instances, circulating anticoagulants

MATERIALS AND METHODS

Techniques employed for the routine coagulation studies have been published elsewhere.[9] Whole blood was anticoagulated with 0.1 M sodium oxalate (1 part oxalate to 9 parts blood) or with dried balanced oxalate. Plasma was defibrinated with bovine thrombin[†] and allowed

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to stand several hours before use. The subsequent addition of bovine fibrinogen to this plasma failed to induce a clot. Bovine thrombin was dissolved in veronal buffer[6] and stored frozen in small aliquots or kept at 4° C. in 50 per cent glycerol. Bovine fibrinogen* was dissolved in saline and used fresh. Fibrinogen concentrations were determined by the method of Ratnoff (normal range, 164 to 415 mg. per cent).[18] Normal or the patient's serum was decalcified with 0.1 M sodium oxalate (1 part oxalate to 9 parts serum).

Plasma Thrombin Time.—The plasma thrombin time was performed by determining the time necessary for the coagulation of 0.4 ml. plasma (anticoagulant: dried balanced oxalate) after 0.1 ml. thrombin (frozen, 3.3 μ/ml.) was added.

Antithrombin Activity.—Antithrombin activity was determined by measuring the ability of 24-hour-old serum (kept in sterile glass tubes at 37° C.) to inactivate a standard quantity of thrombin.

Isolation of Fibrinogen.—Fibrinogen was isolated from plasma by precipitation in phosphate buffer[19] or by the freeze-thaw technique.[20] The absorption of plasma with $Al(OH)_3$ was omitted prior to the phosphate buffer precipitation and the final fibrinogen product was dialyzed for 8 hours. Fibrinogen obtained from 6 ml. of plasma by the freeze-thaw technique was washed 3 times with cold saline and reconstituted in 2 ml. saline.

Ammonium Sulfate Fractionation.—Normal defibrinated plasma† or decalcified serum, both absorbed with barium sulfate, was dialyzed for 48 hours at 4° C. against 33 per cent, then 60 per cent saturated ammonium sulfate solutions. The precipitates were separation by centrifugation, washed 4 times with the respective ammonium sulfate solutions, reconstituted in saline to their original volumes and dialyzed against saline at 4° C. for 48 hours. The supernatants of the 60 per cent ammonium sulfate precipitates were dialyzed against saline in a similar manner.

Viscosity Index.—All viscosity studies were carried out in an Ostwald viscosimeter. The plasma viscosity was determined on a 10 ml. sample (anticoagulant: dried balanced oxalate) at 27.5° C.; the viscosity, compared to 10 ml. distilled water, was expressed as the "viscosity index."

RESULTS

A total of 88 patients were studied. The majority of patients were selected at random; however, toward the latter part of the study patients were selected with hypoalbuminemia.

Plasma Thrombin Time.—The normal plasma thrombin times ranged from 26 to 32 seconds. Prolonged plasma thrombin times were found in nearly all of the diseases studied, including 2 patients in late pregnancy and in 3 out of 4 newborns. Included in the study were hepatocellular liver diseases of many etiologies, acute and chronic leukemias, malignant lymphomas, malignancies, infections, multiple myeloma, collagen diseases, granulomas, metabolic disorders, malnutrition, and renal diseases. The thrombin times were usually increased from 1½ to 2 times over the normal control, but were occasionally prolonged more than 3 to 4 times. Normal thrombin times were encountered in a few patients. No correlation was noted between the thrombin time, platelet count, whole blood clotting time, or plasma prothrombin time.

Fibrinogen Concentration.—The fibrinogen concentrations of the patients ranged from 220 to 1,178 mg. per cent. Over half of the patients exhibited moderately elevated fibrinogen concentrations. Normal fibrinogen concentrations were found in the plasmas of the newborn and slightly elevated levels were obtained on women late in pregnancy. In no instance was hypofibrinogenemia observed Although a wide range of plasma thrombin times was found at any one fibrinogen concentration, an increase in plasma thrombin times could be correlated roughly with increase in fibrinogen concentration (Fig 1) Many plasma thrombin times were prolonged in the presence of normal fibrinogen concentrations, but at no time were normal thrombin times observed in the presence of elevated fibrinogen levels

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†Fibrinogen was removed from plasma by the addition of thrombin.

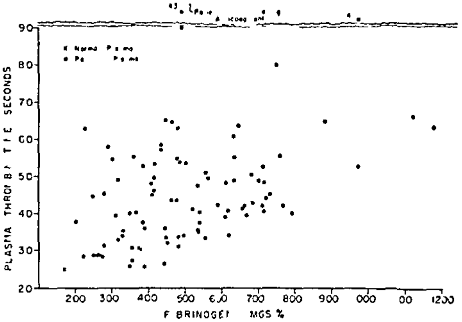

Fig 1—The relationship of the plasma thrombin time to fibrinogen concentration With increasing plasma fibrinogen concentration there is a tendency toward a corresponding rise in plasma thrombin time ($r = 0\ 29$ $p < 0\ 01$)

Antithrombin Activity—Essentially normal antithrombin activity was found in most of the sera examined, but some exhibited moderate increases or decreases (Fig 2) Two sera with increased antithrombin activity were from patients receiving Dicumarol The plasmas of patients with increased antithrombin activity did not demonstrate greater prolongation of their thrombin times than the plasmas of patients associated with normal antithrombin activity In the presence of decreased antithrombin activity the plasma thrombin times were still prolonged

Plasma Viscosity—Increased plasma viscosity was found in many of the patients studied and correlated better with elevation of the fibrinogen than with the plasma globulin level In the majority of plasmas the increase in viscosity was associated with elevation of both the plasma fibrinogen and globulin, but in a few, with either hyperfibrinogenemia or hyperglobulinemia alone Normal or only slightly increased fibrinogen or globulin concentrations were found in plasmas with normal viscosities In no instance was elevation of the plasma viscosity associated with both normal fibrinogen and globulin concentrations In general, the plasma viscosity observed in diseases other than multiple myeloma was slightly to moderately elevated, while more pronounced increases in viscosity were found in multiple myeloma and in a patient with lymphosarcoma, in the latter instances, the abnormal globulins demonstrated in the plasma were characterized by narrow, tall, sharp gamma, "M" or beta globulin peaks on electrophoresis

A wide range of plasma thrombin times was found at any one viscosity index. Although a correlation was suggested between the slight to moderate increase in plasma viscosity and prolongation of the thrombin time (Fig. 3), extreme increases in plasma viscosities observed in patients with multiple myeloma and in the patient with lymphosarcoma were not accompanied by proportional increases in the thrombin times. Many plasmas with prolonged thrombin times were found to have normal viscosities, yet their thrombin times were improved by the addition of normal defibrinated plasma. Normal plasma thrombin times were not observed in the presence of abnormally high plasma viscosities. Normal plasma viscosities were found in the 2 patients in late pregnancy and in the 4 newborns.

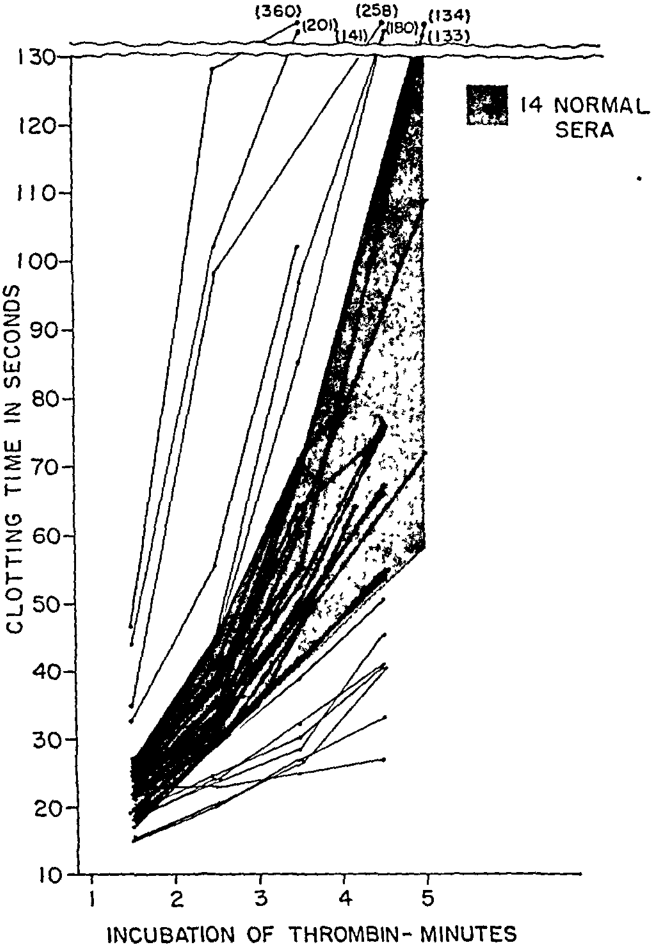

Fig. 2.—Antithrombin activity. 0.8 ml. thrombin (6.6 μ/ml.) was incubated with 0.8 ml of the patient's serum and, at the intervals indicated, 0.4 ml. of the mixture was withdrawn and added to 0.2 ml. bovine fibrinogen (300 mg. per cent) and the clotting time was then determined. Normal antithrombin activity is present in the majority of sera with few demonstrating increased or decreased activity.

In vitro, a correlation between increasing viscosity and prolongation of the clotting of fibrinogen by thrombin could be demonstrated by the addition of polyvinylpyrrolidone or increasing concentrations of isolated bovine or human fibrinogen (Fig 4, A to C) In a patient with multiple myeloma, a reduction in temperature resulted in marked increase in the plasma viscosity and corresponding increase in the plasma thrombin time of greater degree than that observed in the normal control plasma (Fig 4, D)

Demonstration of Normal Clotting Reactivity of the Isolated Fibrinogen.—Fibrinogen isolated from pathologic plasmas with prolonged thrombin times clotted as readily as that obtained from normal plasmas upon the addition of thrombin (Fig 5) As the concentration of the patient's or normal isolated fibrinogen increased, there was a gradual prolongation of the clotting time of the fibrinogen by thrombin with decreasing concentration of the isolated fibrinogen below a 100 mg per cent, a more abrupt increase in clotting time of the fibrinogens by thrombin resulted The fibrinogen from an occasional plasma failed to precipitate out by the freeze thaw method Qualitative abnormalities could not be excluded in the fibrinogens of these patients The isolated fibrinogen of only one of the four plasmas from newborns was studied and found to be normal (the thrombin time of this newborn plasma was normal)

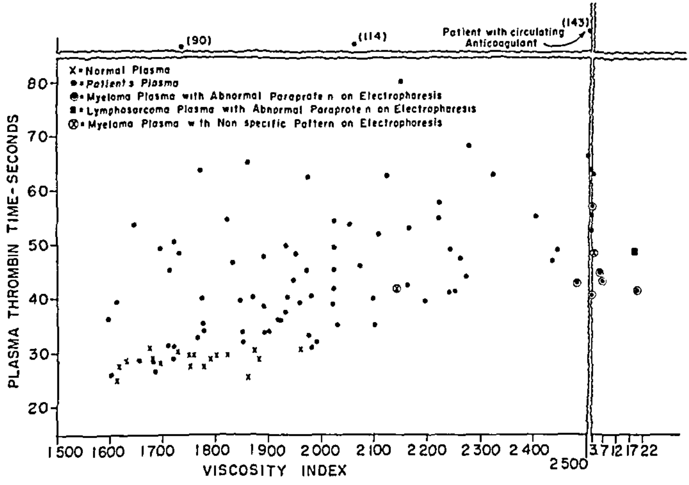

Fig 3.—The relationship of plasma thrombin time and plasma viscosity. A correlation is suggested between the plasma thrombin time and slight increases in plasma viscosity. However extreme elevation in viscosity is not accompanied by proportional rise in thrombin time. No over-all correlation was found between the plasma thrombin time and viscosity on statistical analysis.

Failure of Normal Platelet Suspensions to Correct the Prolonged Plasma Thrombin Time.—The addition of normal platelet suspensions to the patient's plasma in equal volumes failed to correct the prolonged thrombin time of the latter

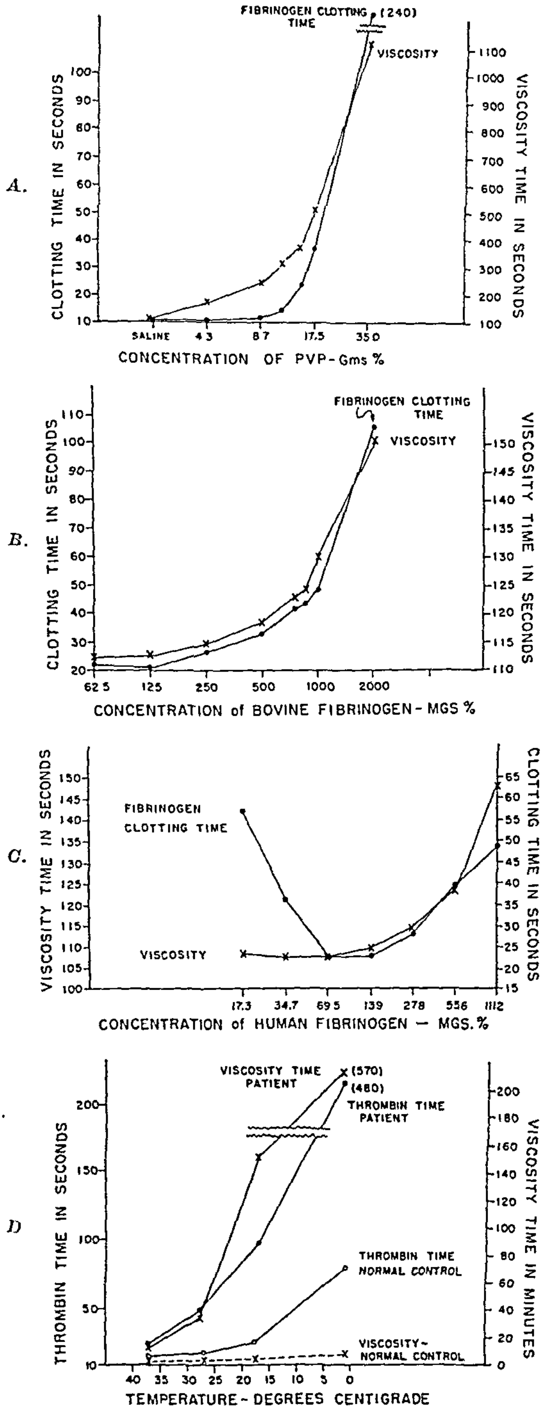

Fig. 4.—The influence of increasing viscosity on the bovine fibrinogen and plasma thrombin times. *A*, The effect of increasing concentration of polyvinylpyrrolidone (PVP) on the viscosity and clotting time of a PVP-bovine fibrinogen mixture. To 0.2 ml. bovine fibrinogen (300 mg. per cent) and 0.2 ml. PVP dissolved in distilled water in the concentration indicated. 0.1 ml. thrombin (6.6 μ/ml.) was added. The viscosity of 5 ml. of the mixture was determined at 25° C. *B*, The effect of increasing concentration of bovine fibrinogen on the viscosity and thrombin clotting time. The 0.4 ml. bovine fibrinogen dissolved in saline in the concentration indicated, 0.1 ml. thrombin was added as above. Viscosity was determined as in *A*. *C*, The effect of increasing concentration of human fibrinogen on the viscosity and thrombin clotting time. Human fibrinogen was isolated from normal plasma by the freeze-thaw technique and studied in similar manner as *B*. *D*, The effect of decreasing temperature on the plasma thrombin time and viscosity in a patient with myeloma.

Effect of Dilution of Plasma on the Prolonged Thrombin Time—Progressive serial dilution of the plasmas with saline to 1 64 generally led to a gradual prolongation of the plasma thrombin times (Fig 6) A transient shortening of the thrombin time was associated with the plasmas exhibiting higher fibrinogen levels while the dilution curves of plasmas with more normal fibrinogen levels were similar to the dilution curves of the normal control plasmas This phenomenon could be reproduced in vitro by varying the fibrinogen level of the plasma to be diluted Normal defibrinated plasma to which was added bovine fibrinogen to a concentration of 2,000 mg per cent showed transient shortening of the thrombin time on dilution with saline A bovine fibrinogen level of 200 mg per cent in the normal defibrinated plasma resulted in a more normal type of dilution curve In one patient with far advanced cirrhosis, marked shortening of the prolonged plasma thrombin time occurred upon dilution of his plasma despite only slight elevation of the fibrinogen level (492 mg per cent) (Fig 6)

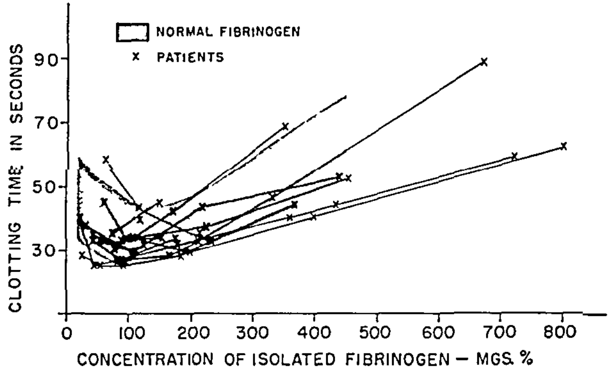

Fig 5—Demonstration of normal clotting reactivity of the isolated fibrinogen To 0 4 ml of the isolated fibrinogen dissolved in saline 0 1 ml thrombin (1 1 μ/ml) was added and the clotting time determined The fibrinogens obtained from pathologic plasmas clotted as readily as those obtained from normal plasmas

In this patient, the plasma formed a jellylike clot upon the addition of thrombin, the plasma viscosity was slightly increased (viscosity index 2 514), the antithrombin activity decreased, serum globulin elevated (6 2 Gm per cent), and the whole blood clotting time prolonged The addition of 1 part of the patient's plasma to 9 parts of normal plasma resulted in prolongation of the thrombin time of the latter A circulating anticoagulant was felt to be present in this patient inhibiting the conversion of fibrinogen to fibrin

The Corrective Effect of Normal Whole or Defibrinated Plasma or Serum on the Prolonged Plasma Thrombin Time—

The addition of normal whole or defibrinated plasma The addition of 1 part of the patient s whole or defibrinated plasma to 9 parts of normal plasma did not significantly prolong the thrombin time of normal plasma over that produced by the addition of saline. One part of normal whole plasma consistently improved the prolonged plasma thrombin time of 9 parts of the patient's plasma.

The addition of normal defibrinated plasma in equal or one-tenth the volume amounts to the patient's plasma resulted in significant reduction in the prolonged thrombin time in almost all of the plasmas studied, irrespective of the disease process (Fig. 7). The effect was more pronounced when equal volumes of normal defibrinated plasma were employed. In most instances the abnormal thrombin time did not quite return to normal, although some plasmas with only slight prolongation of the thrombin times returned completely to normal. In contrast the addition of an equal volume of saline resulted in further increase in the majority of the samples. The mixture of equal amounts of (a) two abnormal whole plasmas with prolonged thrombin times, (b) one abnormal plasma defibrinated and another whole plasma with a prolonged thrombin time, or (c) a pathologic plasma defibrinated and its own whole plasma failed to correct each other, although all were shortened separately by the addition of normal defibrinated plasma. Normal plasma defibrinated by heating at 56° C. for 3 minutes

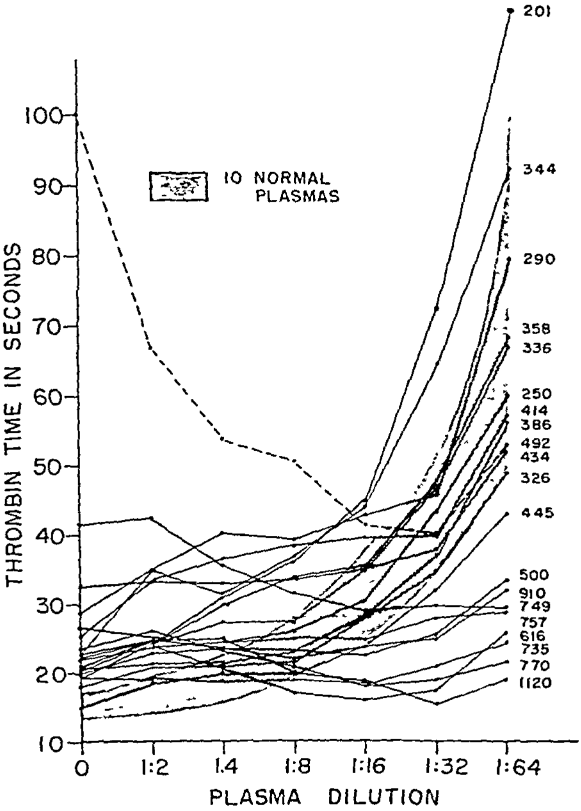

Fig. 6.—Effect of dilution on the prolonged plasma thrombin time. To 0.4 ml. of the patient's plasma (anticoagulant: 0.1 M sodium oxalate) serially diluted with saline to 1:64, 0.1 ml. thrombin (6.6 μ/ml.) was added and the clotting time determined. The number to the right of each curve represents the fibrinogen concentration of the undiluted plasma. The plasma with the circulating anticoagulant is indicated in the broken line.

still possessed corrective effect The prolonged thrombin times of 2 of the 3 newborn cord plasmas were not shortened by the addition of equal amounts of normal defibrinated plasma, but in the third newborn there was improvement The slightly prolonged plasma thrombin times in late pregnancy were normalized by the addition of normal defibrinated plasma

The addition of normal serum Normal serum in equal or one tenth the volume amounts produced even greater shortening than comparable quantities of normal defibrinated plasma, returning many of the abnormal thrombin times to normal (Fig 7) The accelerating effect of serum was partially diminished by incomplete decalcification with sodium oxalate (1 part oxalate to 9 parts serum) After decalcification, the accelerating effect of serum approximated that of normal defibrinated plasma Normal serum produced a slight shortening of the normal plasma thrombin time, but the effect could be eliminated by decalcification Decalcified sera obtained from patients with prolonged plasma thrombin times exhibited little or no accelerating effect on plasmas with prolonged thrombin times

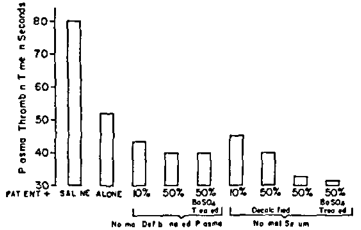

Fig 7—The corrective effect of normal defibrinated plasma and serum on the prolonged plasma thrombin time The values indicated represented the average of 26 patients studied The various reagents indicated along the abscissa were added to the patient's plasma in equal or one tenth the volume amounts To 0 4 ml of the mixture or patient's plasma 0 1 ml thrombin (3 3 μ/ml) was added and the clotting time determined The normal control thrombin time ranged from 26 to 32 seconds

Physicochemical study of the accelerator under investigation The accelerator effect in normal defibrinated plasma or serum was not decreased by dialysis barium sulfate absorption, freezing, prior removal of platelets from plasma by centrifugation, factor V depletion (by heating at 37° C for 24 hours), or *Dicumarol administration Of the 2 newborn cord plasmas studied both showed* no reduction in the accelerator effect

The thrombin time of normal whole plasma was not influenced by barium sulfate absorption, dialysis, removal of platelets by centrifugation, clotting in glass or siliconized tubes, or by Dicumarol administration The addition of an equal volume of normal defibrinated plasma to whole normal plasma did not shorten the thrombin time of the latter, the result excludes the possible activation in normal plasma of an accelerator substance by thrombin Mixtures of equal volumes of normal plasmas gave values no shorter than each tested separately, thereby excluding the possibility of nonspecific acceleration of the thrombin time due to mixtures of foreign plasmas

Plasmas from patients deficient in antihemophilic globulin, plasma thromboplastin component, plasma thromboplastin antecedent, Hageman trait, and from a patient with agammaglobulinemia exhibited normal thrombin times and no reduction in the accelerator effect when tested on pathological plasmas with delayed thrombin times.

Heat Stability.—The thrombin time of normal or pathologic plasmas increased only slightly when the plasma stood at room temperature for 9 hours. Normal sterile plasmas incubated in glass tubes at 37° C. displayed a gradual moderate increase in thrombin times after many days' storage. The accelerator effect was still demonstrable in normal plasma after storage at 37° C. for 77 days and in normal decalcified serum kept at 4° C. for 56 days. Heating normal defibrinated, barium sulfate-treated plasma or decalcified serum at 60° C. for up to 60 minutes resulted in gradual loss of activity when tested on the pathologic plasmas; the heated normal defibrinated plasma when added to normal plasma in equal amounts actually resulted in increase in the thrombin time of the latter.

Ammonium Sulfate Fractionation.—Normal defibrinated plasma and decalcified serum, both absorbed with barium sulfate, were subjected to ammonium sulfate fractionation. The 0 to 33 per cent ammonium sulfate precipitates obtained from plasma or serum demonstrated no accelerator effect, the 33 to 60 per cent precipitates revealed slight accelerator effect, and the supernatant of the 33 to 60 per cent precipitates showed maximal accelerator effect. None of the plasma or serum ammonium sulfate precipitates contained significant thrombin activity. Thrombin added to these precipitates produced no fibrin clots. On paper electrophoresis the 0 to 33 per cent precipitates contained gamma globulin only; the 33 to 60 per cent precipitates, mainly gamma, beta, and alpha globulins with 6.9 to 8.5 per cent albumin; the supernatant of the 33 to 60 per cent precipitates had approximately 84 to 94 per cent albumin with small amounts of alpha and beta globulins.

Plasma from a patient with cirrhosis and a moderately prolonged plasma thrombin time was similarly subjected to ammonium sulfate fractionation. No accelerator effect was found in the 0 to 33 per cent and 33 to 60 per cent precipitates, and markedly diminished accelerator effect in the supernatant of the 33 to 60 per cent precipitate. On paper electrophoresis, similar electrophoretic components were found in the various ammonium sulfate precipitates and supernatant, with an increase in all the globulin fractions and a marked decrease in the albumin fraction.

The supernatant of the 33 to 60 per cent ammonium sulfate precipitates of normal plasma and serum exhibited moderate loss of activity after they were heated at 60° C. for 30 minutes.

Plasmas from one patient with disseminated lupus erythematosus and from one with malnutrition (serum albumin 1.6 and 1.7 Gm. per cent, respectively) were subjected to similar barium sulfate absorption and ammonium sulfate fractionation. The supernatants of the 60 per cent precipitates, containing mainly albumin, were lyophilized and reconstituted in smaller volumes of saline approximating the albumin concentration of the normal control (serum albumin 4.3 Gm. per cent). The concentrated albumin preparations from the patients exhibited some accelerator effect but to a lesser degree than the normal control albumin. Although concentration of the albumin had been achieved, the effect was partly abolished by the concomitant increase in antithrombin activity in the albumin preparation.

*Cohn's Plasma Fractions**—Cohn's plasma fractions were dissolved in saline in 3 Gm. per cent concentration and added to the pathologic plasmas in equal volumes. The accelerator effect was found mainly in fraction V with slight activity in fraction IV-4. No activity was found in fractions I, II, III, IV-1, and IV-7. Actually, fractions I, II, and III demonstrated an inhibitory effect on the thrombin time of the pathological plasmas. On paper electrophoresis, fraction V contained almost 100 per cent albumin, fraction IV-4, 33 per cent albumin with smaller amounts of alpha, beta, and gamma globulins and fraction IV-1 only trace amounts of albumin with larger amounts of alpha, beta, and gamma globulins. Albumin was not present in detectable amounts in the remaining Cohn fractions.

Electrophoretic Separation of Normal Plasma and Serum.—By the technique of paper electrophoretic separation, the accelerator effect in normal de*fibrinated, barium sulfate treated plasma and normal decalcified serum* was localized mainly in the albumin component with slight accelerator effect noticeable in the alpha 1 and gamma 1 globulins.

The Accelerator Effect of Transfused Normal Whole Blood in Vivo.—The infusion of one unit of normal whole blood (17 days old, kept at 4° C.) to a patient with aplastic anemia and hypoalbuminemia resulted in significant improvement in the prolonged plasma thrombin time over a period of 4 days during which observations were made.

The Effect of Commercial Gamma Globulin, Bovine and Human Albumin, and Acacia on the Plasma Thrombin Time.—

Gamma globulin. Gamma globulin (immune human globulin) in 1 Gm. per cent concentration displayed no accelerator effect on the prolonged plasma thrombin time in vitro.

Albumin. Bovine† or human albumin in 4.25 Gm. per cent concentrations demonstrated even more marked accelerator effect than normal defibrinated plasma in vitro. The effect was not reduced by decalcification with oxalate, but diminished to a slight extent when the preparations were heated at 60° C. for *30 minutes. The infusion of 100 ml. of a 25 per cent concentration human salt* poor albumin‡ into one patient with severe cirrhosis, hypoalbuminemia (serum albumin 1.5 Gm. per cent), and marked ascites and into another patient in the nephrotic phase of chronic glomerulonephritis (serum albumin 2.0 Gm. per cent) with anasarca, resulted in impressive though transient improvement in the prolonged plasma thrombin times (Fig. 8).

Acacia: Acacia in varying concentrations from 0.56 to 4.5 Gm. per cent exhibited more pronounced accelerator effect in vitro than comparable quantities of normal defibrinated plasma on the prolonged plasma thrombin time. Shortening of the thrombin time also occurred when acacia was added to normal plasma. Heating acacia at 60° C. for 30 minutes or decalcification with sodium oxalate resulted in slight loss of accelerator effect.

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†The Armour Laboratories Chicago Ill.

‡Courtland Laboratory, Los Angeles Calif.

The Lack of Corrective Effect of Normal Defibrinated Plasma Made Hypoalbuminemic by Saline on the Prolonged Plasma Thrombin Time.—Normal defibrinated plasma made hypoalbuminemic by dilution with saline (serum albumin 1.4 Gm. per cent) was as ineffective in improving the prolonged plasma thrombin time as plasma from a patient with cirrhosis and a serum albumin concentration of 1.7 Gm. per cent.

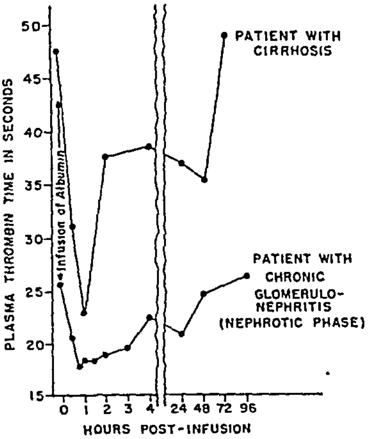

Fig. 8.—The in vivo corrective effect of intravenous infusions of human albumin on the prolonged plasma thrombin time. 100 ml. of 25 per cent human salt-poor albumin was administered intravenously over a 20-minute period and the plasma thrombin times (0.1 ml. glycerol-thrombin, 5 μ/ml., added to 0.4 ml. plasma) determined at the intervals indicated following the infusions. Note the rapid, but transient corrective effect of the infused albumin on the prolonged plasma thrombin times of the patients.

Correlation of the Prolonged Plasma Thrombin and Bovine Fibrinogen Clotting Times With the Serum Albumin Level.—The increase in plasma thrombin time could be correlated with the serum albumin level. The patients with normal or slightly decreased serum albumin levels demonstrated normal or slightly prolonged plasma thrombin times. With decreasing serum albumin levels, the plasma thrombin times became increasingly prolonged (Fig. 9, *A*). No correlation was evident between the plasma thrombin times and serum globulin levels.

The clotting time on the addition of thrombin to a mixture of bovine fibrinogen with the patient's defibrinated plasma or bovine albumin could be correlated with the albumin level of the mixture. Depression of the albumin level resulted in prolongation of the bovine fibrinogen clotting time (Fig. 9, *B*).

DISCUSSION

The results of this study confirm many of the previous observations in which prolonged plasma thrombin times were reported in a variety of disease states. Prolongation of the plasma thrombin time appeared to be due to a multiplicity of factors, often occurring in combination. The most constant abnormality appeared to be a deficiency of a substance in plasma aiding the conversion of fibrinogen to fibrin In addition, an increase in fibrinogen concentration also seemed to contribute to the delayed thrombin time in many of these patients Hypofibrinogenemia was not observed in the present series The antithrombin activity did not generally seem to correlate with the *prolonged plasma thrombin time* although increases in antithrombin were found in a few patients and may

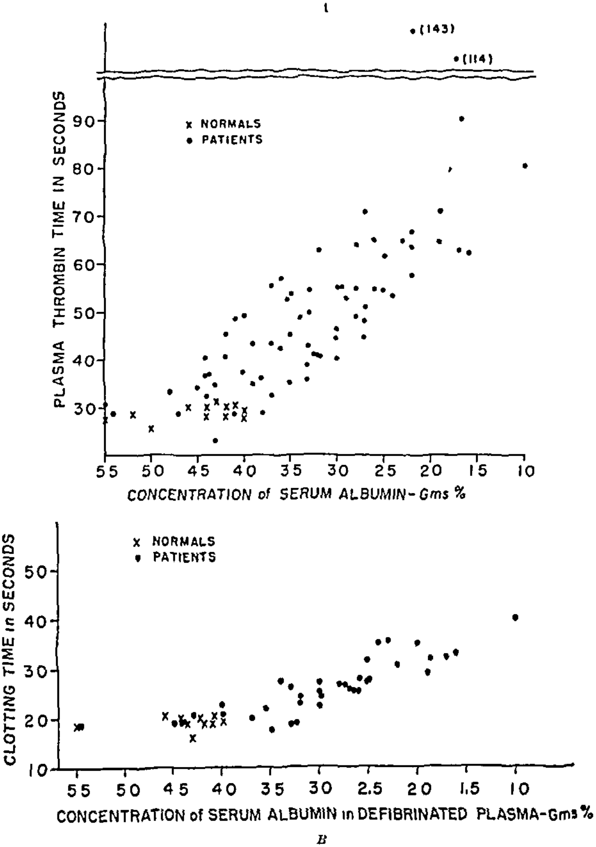

Fig 9—The effect of the concentration of albumin on the plasma thrombin and bovine
fibrinogen clotting times *A*, concentration on the
plasma thrombin time With note the gradual in
crease in plasma thrombin ti concentration of the
patient a mixture of 0 3 ml
of the xalate) and 0 1 ml
bovine added and the clot-
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have contributed to the abnormality. Only a single instance of circulating anticoagulant was recognized. Abnormal clotting reactivity of fibrinogen to thrombin could not be demonstrated in the plasmas studied.

The data presented are in agreement and confirm all observations previously reported on a fibrinogen conversion accelerating effect in normal defibrinated plasma. In addition, the accelerator effect was also demonstrated in normal serum. The greater accelerator effect of serum than plasma is probably due to the known clot-accelerating effect of calcium in serum.[21] The studies in this report also suggest no relationship between platelets or any of the known plasma or serum coagulation factors and the accelerator effect in plasma or serum. Suspensions of normal platelets were shown to possess no accelerator effect on the prolonged plasma thrombin times. No correlation was evident between the platelet count and thrombin time.

The data obtained indicate that the accelerator effect in normal plasma or serum was associated mainly with albumin or a substance related to albumin. The apparent localization of the accelerator effect to albumin was supported by many observations in this study. The increase in plasma and bovine fibrinogen clotting times correlated well with decreasing serum albumin levels; no correlation was evident with serum globulin levels. The ammonium sulfate fractionated precipitates of normal plasma or serum containing the active accelerator effect were associated with the albumin component. While the fractions of normal plasma with maximal activity were different from that previously reported,[6] the difference may have been in the technique of fractionation. By paper electrophoretic separation, the accelerator effect in plasma and serum was confined mainly to the albumin. Pure bovine or human albumin preparations exhibited accelerator effect in vitro and with the latter preparation, also in vivo. The accelerator effect was found primarily in Cohn's plasma fraction V, which contained approximately 99 per cent albumin, and to a slight extent in fraction IV-4, which contained in addition to alpha and beta globulins, 32.9 per cent albumin.

Whether the accelerator effect in albumin is due to a true coagulation factor or to a nonspecific colloidal effect was not resolved. The conversion of fibrinogen to fibrin in vitro is dependent on an optimal colloidal state.[11] Acacia in 2 to 6 per cent concentration can accelerate the conversion of fibrinogen to fibrin probably by providing a more optimal colloidal state. In this report acacia demonstrated a similar accelerating effect in vitro on plasmas with delayed thrombin times. The almost universal tendency for the serum albumin level to fall with acute or chronic disease correlates well with the occurrence of increased plasma thrombin times in many disease processes. The decreased serum albumin levels may result in suboptimal colloidal states. Accelerator effect in normal defibrinated plasma and serum probably acts simply by restoring a suboptimal colloidal state to normal. The moderate loss of accelerator effect in normal defibrinated plasma or decalcified serum after heating could be due to destruction of a coagulation factor in albumin to denaturation of the albumin molecule with impairment of its colloidal effect or to loss of antiheparin activity in pseudoglobulin.[11] The heat stability of the accelerator effect of normal plasma in this report was greater than in the one previous study.[6]

Although the influence of fibrinogen concentration on the clotting time of isolated thrombin fibrinogen mixtures has been well studied, previous work has not emphasized hyperfibrinogenemia as a contributing factor in prolongation of the plasma thrombin time. The present data are in accord with the known tendency of increased concentrations of fibrinogen to clot more slowly. The associated rise in viscosity with increasing concentrations of fibrinogen, whether in plasma or in isolated form may represent an associated secondary phenomenon rather than a direct causative factor in prolongation of the fibrinogen clotting time. While prolongation of the plasma thrombin time by polyvinylpyrrolidone was observed in this study, shortening of the thrombin time by this substance has been reported previously.[22] In addition, the plasma thrombin time in patients with multiple myeloma showed no direct proportional increase with the marked rise in plasma viscosity observed, but seemed to correlate better with the fibrinogen level. An excess of fibrinogen molecules might mechanically interfere with the process of fibrinogen polymerization and fibrin clot formation.

In late pregnancy the slight increase in plasma thrombin time appears to be due to a reduction in albumin and some increase in fibrinogen concentration. The number of newborn plasmas studied in this report is too small to permit definite conclusions, but reduction in serum albumin and possible qualitative changes in the fibrinogen may be important factors in the prolongation of the plasma thrombin time.

Most of the abnormal thrombin times were associated with normal blood coagulation times. No correlation was found between the coagulation time and abnormal thrombin time. In the few patients with prolonged whole blood coagulation times, the thrombin times were not excessively prolonged with one exception. The relative lack of prolongation of whole blood coagulation in the presence of increased thrombin time may be due to the relative shortness of the third stage of coagulation compared to over all coagulation.

The increase in plasma thrombin times did not appear to result in serious hemorrhagic tendencies in the patients studied. The majority of patients were free from clinical bleeding at the time of study. In most of the patients with hemorrhagic manifestations, the thrombin times were not excessively prolonged and other coagulation defects were usually present. The wide spectrum of diseases in which the plasma thrombin time may be prolonged and the relative lack of associated hemorrhagic tendencies in the great majority would suggest the increased plasma thrombin time to have limited clinical significance.

SUMMARY

Prolongation of the plasma thrombin time was found in a wide variety of diseases and in late pregnancy and the newborn. Further study revealed the increase in plasma thrombin time to be a complex phenomenon due to a multiplicity of factors, usually occurring in combination. The most constant abnormality appeared to be a deficiency of a plasma substance aiding the conversion

coagulant. The stage at which factor V is required was not established since platelets free from factor V can only be prepared from blood from a patient with factor V deficiency[17] and such blood was not available to these workers. Hörder and Sokal.[15] however, in a study of a patient with congenital factor V deficiency showed that when normal serum, AHF, and platelets prepared from the patient were preincubated with calcium for 5 minutes the subsequent addition of factor V resulted in complete activation within one minute; they concluded that factor V reacted subsequent to the reaction between AHF, serum factors, and platelets. The experiments to be described confirm this work and indicate that factor V interacts with the sedimentable complex formed by the reaction between AHF, PTC, Stuart factor, and platelets. The present study is confined to the roles of the above named factors and does not attempt to delineate the roles of PTA, Hageman factor, factor X, the Prower factor, or the fourth thromboplastin component.

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Prolongation of the plasma thrombin time was found in a wide variety of diseases and in late pregnancy and the newborn. Further study revealed the increase in plasma thrombin time to be a complex phenomenon due to a multiplicity of factors, usually occurring in combination. The most constant abnormality appeared to be a deficiency of a plasma substance aiding the conversion

of fibrinogen to fibrin. An increase in plasma fibrinogen appeared to be a contributing factor. Circulating anticoagulant was encountered only once and increased antithrombin activity appeared to play a relatively insignificant role in the delayed plasma thrombin time. The deficient substance in plasma, aiding fibrin clot formation, did not appear to be platelets or any of the known plasma or serum coagulation factors. The data presented suggest that the deficient substance is albumin or a substance related to albumin. Clinically, prolongation of the plasma thrombin time did not appear to result in significant hemorrhagic manifestations.

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THE ROLE OF FACTOR V IN THE FORMATION OF BLOOD THROMBOPLASTIN

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BIGGS, Douglas, and Macfarlane[1] showed that the incubation of $Al(OH)_3$ adsorbed plasma, serum platelets, and calcium results in the formation of a powerful coagulant which they named "blood thromboplastin" The term thromboplastin generation' implies the formation of a final prothrombin converting substance, while this may not necessarily be true, it, nevertheless, forms a useful working hypothesis There are, however, other interpretations of the phenomenon For example, Seegers and his associates[2, 3] believe that the thromboplastin generation test measures the transformation of plasma AC globulin (factor V, proaccelerin) to serum AC globulin

It was originally believed by Biggs, Douglas, and Macfarlane[4] that platelets, antihemophilic factor (AHF), factor V, factor VII (serum prothrombin conversion accelerator, SPCA or proconvertin), and Christmas factor (plasma thromboplastin component PTC) together with calcium were necessary for a normal thromboplastin generation test Factor VII is now known to be unnecessary[5, 6] but the other findings of these workers have been confirmed Several 'new" clotting factors essential for thromboplastin generation" have recently been described, these include the Hageman factor,[7] the Prower[8] and Stuart factors,[9, 10] factor X[5] and probably plasma thromboplastin antecedent (PTA)[11] and the fourth thromboplastin component[12] It is extremely unlikely that all these factors react together in a single high order reaction but more probable that 'blood thromboplastin" is the end product of a series of intermediate reactions[4, 13, 14] These reactions may be exceedingly complicated and Seegers[3] has stated that "the thromboplastin *generation test is practically as complicated as the coagulation of whole blood* and equally difficult to interpret"

There is evidence that AHF and serum interact forming an intermediate product which has been referred to as Product I[13, 15, 16] It must be stressed that this term is used for convenience only since it has not been shown or claimed that this intermediate product is the first one formed, that only a single reaction occurs when calcium and AHF are incubated with serum or that only these factors are involved in the formation of this product It appears from the work of Bergsagel and Hougie[13] that *Product I reacts with the* thromboplastic factor of platelets (platelet factor III) forming a sedimentable

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*Present address Department of Clinical Pathology University of Virginia Charlottesville Va coagulant. The stage at which factor V is required was not established since platelets free from factor V can only be prepared from blood from a patient with factor V deficiency[17] and such blood was not available to these workers. Hörder and Sokal,[18] however, in a study of a patient with congenital factor V deficiency showed that when normal serum, AHF, and platelets prepared from the patient were preincubated with calcium for 5 minutes the subsequent addition of factor V resulted in complete activation within one minute; they concluded that factor V reacted subsequent to the reaction between AHF, serum factors, and platelets. The experiments to be described confirm this work and indicate that factor V interacts with the sedimentable complex formed by the reaction between AHF, PTC, Stuart factor, and platelets. The present study is confined to the roles of the above named factors and does not attempt to delineate the roles of PTA, Hageman factor, factor X, the Prower factor, or the fourth thromboplastin component.

METHODS AND MATERIALS

All actual clotting tests were performed in a 37° C. water bath in uncoated tubes. Silicone-coated syringes and glassware were used for collection of blood unless otherwise required by a specific method.

A patient with a congenital deficiency of factor V, a patient with Stuart factor deficiency, a severe hemophiliac (less than 3 per cent AHF), and a patient with Christmas disease were used as subjects in these experiments. The patient with the congenital deficiency of factor V (proaccelerin) has been previously reported and has been found to have 3 per cent factor V.[19] The patient with the Stuart factor deficiency has also been previously studied[8, 20] and the Stuart factor content of this patient's serum is less than 1 per cent when measured by the thromboplastin generation test.

Whole blood was collected in glass tubes and allowed to clot and stand at 28° C. for 24 hours before the serum was separated. Sera from the patient with Christmas disease and from the patient deficient in Stuart factor were treated for ½ hour with barium sulfate (50 mg. per ml.) which adsorbs Stuart factor and PTC but not factor V. The serum factors were subsequently eluted from the $BaSO_4$ by 5 per cent sodium citrate. The two preparations were then dialyzed overnight against 0.85 per cent saline and the volumes finally adjusted to the original volumes of serum. Platelets from the patient with the congenital deficiency of factor V were prepared by the technique used in the thromboplastin generation test.[21] The platelets were incubated with a variety of coagulation factors and then sedimented by centrifuging at 25,000 g for 5 minutes. The supernatant was discarded, the sediment washed once with saline and then centrifuged at 25,000 g for 5 minutes. After discarding the supernatant, the deposit was suspended in a volume of saline equal to the volume of saline in which the platelets were originally suspended. The imidazole buffer, pH 7.25, used in the reaction mixtures was prepared by the method of Mertz and Owen.[22]

RESULTS

Failure of Alumina-Treated Plasma From a Patient With Congenital Deficiency of Factor V to Form Thromboplastin.—It has been shown previously that $Al(OH)_3$-adsorbed plasma from a patient with a congenital deficiency of factor V is defective in the thromboplastin generation test but the serum is normal in respect of this test.[18] This work was confirmed (Fig. 1). The finding that substitution of normal platelets for the patient's platelets when the patient's $Al(OH)_3$-adsorbed plasma is used results in a slight but significant improvement in "thromboplastin generation"[18] was also confirmed (Fig. 1).

When normal $Al(OH)_3$ adsorbed plasma, normal serum, and normal platelets were incubated together, the "thromboplastin generated" gave the same clotting time with the patient's substrate plasma as normal plasma.

Effect of Factor V on Product I, Platelet Reaction.—In the following experiment (Table I) the barium sulfate eluates of the sera deficient in Stuart factor and PTC (diluted 1:5) were used as sources of PTC and Stuart factor, respectively, while the $Al(OH)_3$ adsorbed plasmas (diluted 1:5) from the hemophiliac and patient with factor V deficiency were used as sources of factor V and AHF, respectively. An incubation mixture of any 3 of these reagents contains all the coagulation factors present in normal serum and normal $Al(OH)_3$ adsorbed plasma with the exception of only one coagulation factor (see Table I). The 4 possible combinations of groups of 3 of the 4 reagents and a normal control mixture containing all 4 reagents were separately preincubated together in the presence of calcium for 5 minutes. Platelets from a patient with a congenital deficiency of factor V were then added to each of the 5 incubation mixtures and these were then further incubated for 5 minutes. The platelet material was sedimented by centrifuging, washed once and resuspended a volume of saline equal to the volume of saline in which the platelets were originally suspended. Two types of experiments were performed on the suspensions of platelet sediment. Approximately half of each suspension was diluted 1:3 with 0.85 per cent saline and the relative coagulant activity of each diluted suspension determined by adding 0.1 ml. of the suspension under test together with 0.1 ml. 0.025 M $CaCl_2$ to 0.1 ml. of a normal plasma substrate. The clotting times of the substrate plasma are shown in Table I under Column 3. In the second type of experiment 0.2 ml. of the remaining half of each suspension of platelet sediment was incubated with 0.2 ml. of the coagulation factor which was omitted in its preparation and 0.2 ml. 0.025 M $CaCl_2$. At minute intervals after the final addition of calcium, the relative coagulant activity of each incubating mixture was determined by removing a 0.1 ml. sample and adding it together with 0.1 ml. 0.025

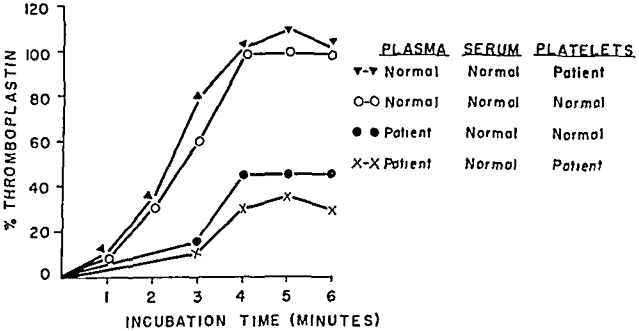

Fig. 1.—Thromboplastin generation test in a case of congenital deficiency of factor V.

M $CaCl_2$ to 0.1 ml. of a normal plasma substrate. It can be seen from Table I that the platelet sediment which was prepared without factor V and had little coagulant activity showed a marked increase in activity after incubation with factor V. The maximum activity was reached after 3 minutes and was equal to that of the normal control sediment prepared using all the coagulant factors. In the case of the remaining sediments no significant increase in coagulant activity occurred when these were further incubated either with the coagulant factor omitted in their preparation or with a factor V source, viz., $Al(OH)_3$-adsorbed hemophilic plasma. Similarly no further coagulant activity resulted when the normal control platelet sediment, which was prepared with all the coagulant factors, was further incubated with factor V.

TABLE I. COMPARATIVE COAGULANT ACTIVITY OF PLATELET SEDIMENTS PREPARED FROM PLATELETS FROM CASE OF CONGENITAL FACTOR V DEFICIENCY

REAGENTS INCUBATED WITH 0.025 M $CaCl_2$, 0.6 ML. PLATELET SUSPENSION, 0.6 ML. IMIDAZOLE BUFFER	CLOTTING FACTOR ABSENT DURING THROMBOPLASTIN GENERATION	COAGULANT ACTIVITY OF PLATELET SUSPENSION			
		SUSPENDED IN SALINE		AFTER 3 MINUTES' INCUBATION WITH COAGULATION FACTOR WHICH WAS OMITTED FROM PREINCUBATION MIXTURE	
		SUBSTRATE CLOTTING TIME	PER CENT ACTIVITY	SUBSTRATE CLOTTING TIME	PER CENT ACTIVITY
		(1) sec.	(2)	(3) sec.	(4)
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	None	30	100	-	-
0.6 ml. eluate of Stuart deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al.(OH)_3$-adsorbed factor V deficient plasma	Stuart factor	79	7	71	11
0.6 ml. eluate of PTC deficient serum 0.3 ml. $Al(OH)_3$-adsorbed hemophilic plasma 0.3 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	PTC	71	11	70	12
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.6 ml. $Al(OH)_3$-adsorbed hemophilic plasma	AHF	90	2	84	5
0.3 ml. eluate of Stuart deficient serum 0.3 ml. eluate of PTC deficient serum 0.6 ml. $Al(OH)_3$-adsorbed factor V deficient plasma	Factor V	72	10	30	100

The clotting times obtained in the above experiment were converted to per cent activity from a thromboplastin dilution curve which was obtained in the following manner. The normal control suspension of platelet sediment was diluted 1:3 in saline and this dilution arbitrarily chosen to represent 100 per cent activity. Serial dilutions were made of this suspension and the coagulant activity of 0.1 ml. of each dilution determined. Thus 0.1 ml. of a dilution was added simultaneously with 0.1 ml. M CaCl$_2$ to 0.1 ml. normal plasma and the clotting time of the mixture determined. A dilution curve, relating clotting time of the mixture to the strength of each dilution added to the clotting mixture was then prepared.

Comparison of One Stage "Prothrombin Times" of Normal and Factor V Deficient Plasma Using Product I Treated Platelets Prepared With and Without Factor V as Thromboplastin Source.—Product I treated platelet sediments were prepared by preincubating for 5 minutes 0.2 ml. normal serum diluted 1:10 (source of both PTC and Stuart factor), 0.2 ml. imidazole buffer and 0.2 ml. 0.025 M CaCl$_2$ with 0.2 ml. of 1:5 dilution of each of the following: (a) normal Al(OH)$_3$ adsorbed plasma and (b) Al(OH)$_3$ adsorbed plasma from patient deficient in factor V. To each of the 2 mixtures was then added 0.8 ml. of a platelet suspension from the patient with factor V deficiency and incubation carried out for a further 5 minutes. The subsequent technique was as described in the section on methods. The 2 final suspensions of platelet sediments were diluted 1:3 in 85 per cent saline. The one stage "prothrombin times" of normal plasma and plasma from the patient with congenital deficiency of factor V were then determined using the sediments prepared with and without factor V as the source of thromboplastin.

TABLE II. MODIFIED ONE STAGE "PROTHROMBIN TIMES" OF NORMAL AND FACTOR V DEFICIENT PLASMAS USING PLATELET SEDIMENTS PREPARED FROM PLATELETS FROM CASE OF CONGENITAL FACTOR V DEFICIENCY

NORMAL SERUM, PATIENT'S PLATELETS IMIDAZOLE BUFFER, CaCl$_2$ INCUBATED WITH	ONE STAGE "PROTHROMBIN TIMES" (SEC.) USING "PLATELET SEDIMENTS" AS SOURCE OF THROMBOPLASTIN: NORMAL PLASMA SEC.	FACTOR V DEFICIENT PLASMA SEC.
Normal Al(OH)$_3$ adsorbed plasma	24	22
Factor V deficient Al(OH)$_3$ adsorbed plasma	47	95

The results of such an experiment are shown in Table II. It can be seen that the "prothrombin time" of the factor V deficient plasma was normal when the platelet sediment prepared with factor V was used as the source of thromboplastin and that the "prothrombin times" of both the normal and factor V deficient plasma were prolonged when the platelet sediment prepared without factor V was used as the thromboplastin source.

DISCUSSION

The results confirm earlier work[13] that a sedimentable coagulant forms when all the factors essential for "thromboplastin generation" are incubated together. The present work also shows that if any one of the following, AHF, PTC, Stuart factor, or factor V is omitted from the preparation of the sedimentable coagulant, it will have comparatively little activity. Subsequent incubation of the defective sediment with the coagulation factor originally omitted does not result in further increase of coagulant activity, except in the case of factor V. It appears, therefore, that factor V acts subsequent to PTC, Stuart factor, platelets, and AHF on the sedimentable complex formed by these factors. While the possibility that factor V may act earlier under more physiologic conditions of clotting is not excluded by either this work or the previous work of Hörder and Sokal,[18] it is rendered unlikely by indirect evidence. Thus, it has been recently shown by Douglas[23] that when whole blood from a patient with hemophilia, Christmas disease (congenital PTC deficiency), or thrombocytopenia is allowed to clot in a glass tube there is a failure to utilize factor V. These findings suggest that factor V enters the reactions involved in blood clotting at a later stage than AHF, Christmas factor, and platelets. If the factor V had been involved in the earlier stages of thromboplastin formation then its utilization in one or more of these conditions would have been anticipated.

The findings can all be fitted into the following scheme which is similar to that suggested by Macfarlane.[16]

(1) PTC + AHF + Stuart factor + Calcium → Intermediate Product I
(2) Intermediate Product I + Platelets → Intermediate Product II (sedimentable)
(3) Intermediate Product II + factor V → Blood Thromboplastin or final prothrombin converting substance (sedimentable)

Such a hypothesis is necessarily incomplete. It does not take into account the Hageman factor, PTA, factor X, and other factors thought to be important in "thromboplastin generation," nor does it consider inhibitory systems. The scheme, however, agrees in part with the view of Owren[24] that antihemophilic "A" and "B" factors (AHF and PTC) and platelets react to form a product which then reacts with proconvertin (factor VII) to form "convertin" (probably the Product II of the above scheme). "Convertin" then reacts with "accelerin" (derived from factor V) to form blood thromboplastin or "prothrombinase," the final prothrombin-converting substance. Since it is now known that factor VII (proconvertin) is not essential for "blood thromboplastin," it is probable that Owren was considering the Stuart factor rather than factor VII (proconvertin) since these 2 factors have many properties in common and the "specific" proconvertin assay technique of Owren and Aas[25] measures not only proconvertin but Stuart factor and perhaps other factors as well.[8] Owren's view that proaccelerin (factor V) is converted to accelerin by the action of thrombin is generally accepted.[26, 27] It has, however, been previously shown that washed sediments, prepared by the technique used in the present work, do not contain demonstrable thrombin.[13] The thrombin necessary for this transformation of factor V must therefore be derived from traces of prothrombin present in the factor V source ($Al(OH)_3$ adsorbed hemophilic plasma), or be incorporated into the sediments as previously suggested by Spaet and Garner[28] and, therefore not demonstrable by the usual techniques

CONCLUSIONS

Experimental evidence is presented indicating that factor V is not required in the formation of Product I thereby differing in this respect from PTC, Stuart factor and AHF, which are necessary for the formation of this product Product I appears to react with the thromboplastin factor of platelets and this in turn reacts with factor V

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VITAMIN B_{12} BINDING CAPACITY OF NORMAL AND LEUKEMIC SERA

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INTRODUCTION

The possibility of assaying vitamin B_{12} by the use of the green alga *Euglena gracilis* var. *bacillaris* was first demonstrated by Hutner and his associates.[1] Ross[2] described in particular a technique by means of which it is possible to determine the concentration of the vitamin in body fluids. Beard, Pitney, and Sanneman[3] and Beard et al.[4] described an abnormally high concentration of vitamin B_{12} in the patients suffering from acute or chronic granulocytic leukemia and acute monocytic leukemia. Patients suffering from acute or chronic lymphocytic leukemia were described as having normal levels of the substance. These findings were confirmed by a number of investigators.[5, 6] Pitney, Beard, and Van Loon[7] demonstrated also that the sera of patients suffering from chronic granulocytic leukemia can bind more vitamin B_{12} than normal serum. This finding was confirmed by Lear and his associates.[8] The purpose of this paper is to present a method useful for the measurement of the vitamin B_{12} binding capacity of serum and the results obtained with normal and pathological sera.

MATERIALS AND METHODS

The assays of vitamin B_{12} were carried out with the method of Ross[2] with only minor modifications. With this technique, it is possible to differentiate a "bound" form of the vitamin, which becomes available to the microorganism after heating the sera to 100° C, from a "free" form, which is available to the microorganism even before heating. In normal sera, the latter form is usually found in very small amounts. If vitamin B_{12} is added to a normal serum and this mixture is inoculated with *E. gracilis* without previous heating, the growth of the microorganism will be less than it would be if the same amounts of vitamin B_{12} were added to distilled water instead of serum. The interpretation of this phenomenon is that the vitamin B_{12} added to the serum becomes linked with a globulin component,[7] and in this form the substance does not support the growth of *E. gracilis*. Our method for the measurement of the binding capacity of sera was developed on the basis of this hypothesis. Serial dilutions of sera from 1:1 (pure serum) to 1:64 were prepared using sterile distilled water as diluting fluid. To 1 milliliter of each of these dilutions, 1 milliliter of a solution of crystalline vitamin B_{12} containing 800 $\mu\mu g$ of substance per milliliter was added. No incubation of the mixtures was done at this point, but the tubes were left at room temperature during the execution of the other steps of the technique. The concentration of "free" vitamin B_{12} in each of these dilutions was assayed. To obtain it, 0.10 milliliter of each mixture was added to 1.9 milliliters of water and 2.0

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milliliters of double strength medium. The tubes were then incubated for 15 minutes at 56° C. This degree of heating does not liberate any of the bound vitamin B_{12} naturally present in normal sera; its purpose is to decrease the possibility of growth of contaminant fungi in the material.

If the concentration of naturally present "free" vitamin B_{12} in the serum, at the dilutions used for the assays, is zero, the vitamin B_{12} recovered should represent the amount of added substance that the serum did not bind. The amount recovered should be greater at the higher dilutions of sera and should be 100 per cent where the vitamin is added to water instead of serum. The free vitamin B_{12} concentration at these dilutions is actually zero in practically all normal sera, but it is sometimes increased in abnormal sera. In this case, the pre-existing amount of vitamin B_{12} should be subtracted from the value found after the addition of crystalline vitamin B_{12}, and the difference will indicate the amount of added substance which actually remained unbound. To accomplish this, a second set of tubes was prepared, containing the sera treated in the way described above, except that to the sera was added distilled water instead of the solution containing vitamin B_{12}. When vitamin B_{12} could be detected in this set of tubes, the values were subtracted from the corresponding ones of the first set.

Plotting the amounts of vitamin B_{12} recovered against the dilutions of sera (Fig. 1), it is possible to obtain a curve from which the binding capacity of a given serum can be estimated. In Fig. 1, it can be noted that the amounts of vitamin B_{12} recovered were sometimes greater than the amounts added; these insignificant differences are within the limits of error for the method employed. All the assays were carried out in triplicate.

RESULTS

Determinations of total and free vitamin B_{12} serum levels were caried out in 180 normal individuals, 59 cases of miscellaneous diseases, 12 cases of pernicious anemia, and 50 cases of leukemia. The values obtained are shown in Table I. Twenty-four of the normal sera were studied with the method described above in order to determine their binding capacity. The original levels of total vitamin B_{12} in these sera ranged from 218 to 1,024 $\mu\mu$g per milliliter and those of the "free" vitamin B_{12} from 0 to 104. When these normal sera were diluted, only a small percentage of the added vitamin B_{12} became bound. At the lower dilutions of sera, the binding capacity was higher, but some of the added vitamin was always recoverable. In most of the experiments, the vitamin bound was between 50 per cent and 80 per cent (between 400 and 650 $\mu\mu$g per milliliter). Fig. 1 shows the average

TABLE I. SERUM VITAMIN B_{12} IN NORMAL AND PATHOLOGICAL SERA ($\mu\mu$G PER MILLILITER)

NO. OF PATIENTS	DIAGNOSIS	RANGE		AVERAGE	
		TOTAL	FREE	TOTAL	FREE
180	Normal	144-1,080	0-104	387*	25
59	Miscellaneous diseases	22-2,200	0-272	672	38
12	Pernicious anemia	0-50	0	34	0
26	Chronic granulocytic leukemia	2,368-16,600	0-216	7,701	88
9	Acute granulocytic leukemia	300-3,580	0-204	988	71
6	Chronic lymphocytic leukemia	140-760	0-328	416	111
7	Acute lymphocytic leukemia	234-860	42-304	497	145
2	Monocytic leukemia	1,376-1,520	0-872		

*Standard deviation ± 140.

binding capacity curve and the standard deviations obtained with normal sera. The same technique was used for the study of our 26 cases of chronic granulocytic leukemia, before treatment Fig 2 shows the average curve and standard deviations of the results The binding capacity was always very high, and the free vitamin often completely disappeared even when there was less than 10 per cent of the serum present Thirteen of these patients were followed during the course of their disease with repeated assays of vitamin B_{12} Twelve of them were treated with Myleran, 6 mg per day Three patients received a course of x-ray therapy when they became resistant to Myleran therapy. One received only x-ray therapy The total vitamin content of the serum of these patients decreased consistently with the reduction in the white blood count, and

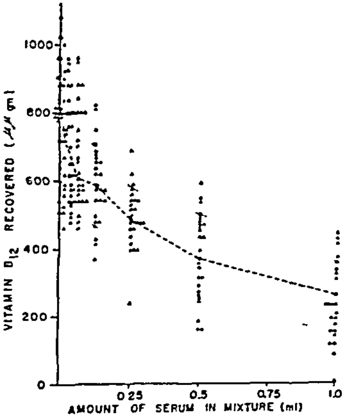

Fig 1

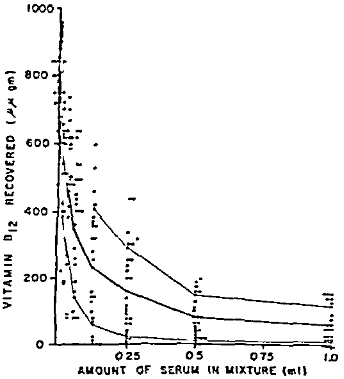

Fig 2

Fig 1.—Vitamin B_{12} binding capacity of 24 normal sera (average curve and standard deviations)

In Figs 1, 2, 4, 5 and 6, the amount of vitamin B_{12} bound is represented by the difference between the amount of vitamin B_{12} added and the amount of vitamin B_{12} recovered

Fig 2.—Vitamin B_{12} binding capacity of 26 sera of patients with chronic granulocytic leukemia. (Average curve and standard deviations)

increased again when the number of leukocytes rose. In Fig. 3 (*A*, *B*, and *C*), the binding capacities of the sera are represented by one value only. This is the amount of vitamin B_{12} bound by the sera at a dilution of 1:8. The choice of the serum binding capacity at this dilution was arbitrary. Fig 4 shows the average curve and standard deviations of the results obtained in these 13 patients when, after a course of Myleran therapy, their white blood count reached normal values In this sera, the total vitamin B_{12} ranged from 350 to 1,860 $\mu\mu$g per milliliter, the "free" from 0 to 216 The results obtained in the untreated chronic granulocytic leukemia are shown in the same figure for comparison Five cases of acute lymphocytic leukemia and 6 of chronic lymphocytic leukemia

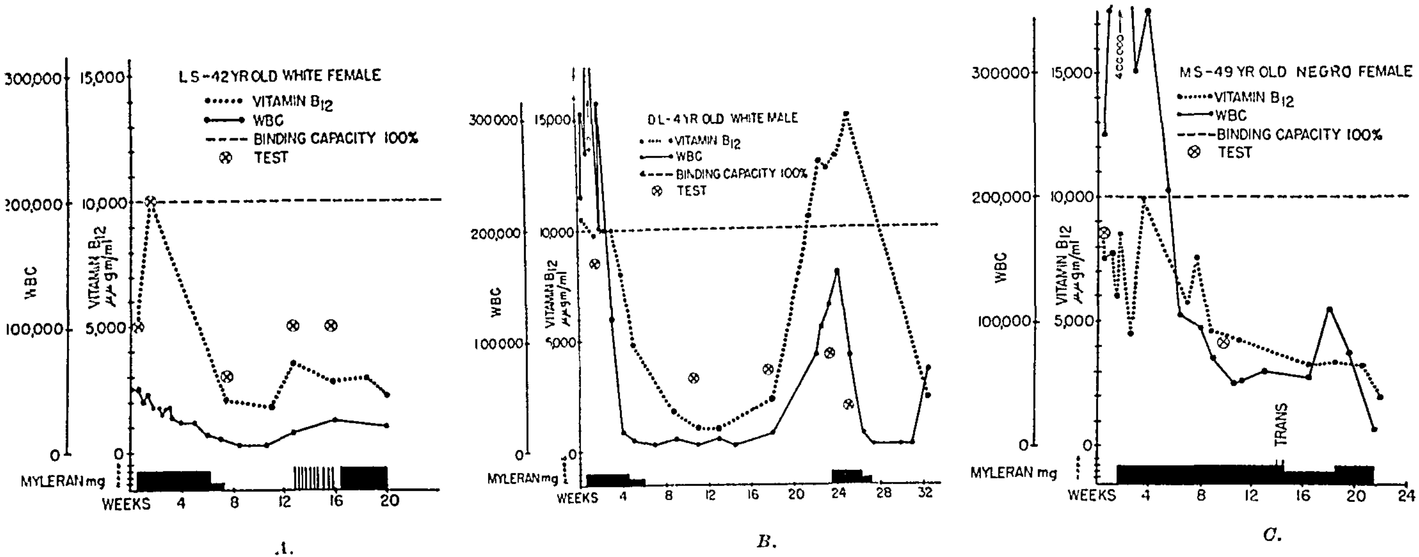

Fig. 3.—A, B, C, Number of leukocytes, serum levels of vitamin B_{12}, and serum vitamin B_{12} binding capacity in three patients with chronic granulocytic leukemia under treatment with Myleran.

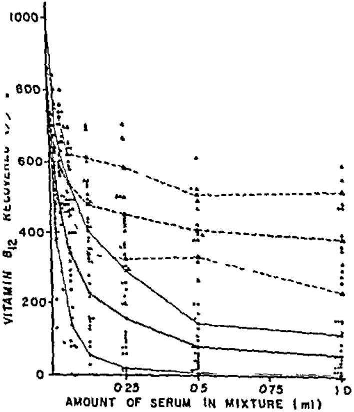

Fig 4

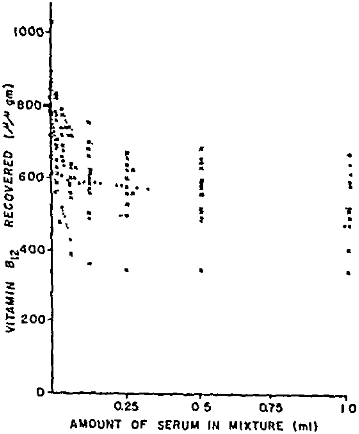

Fig 5

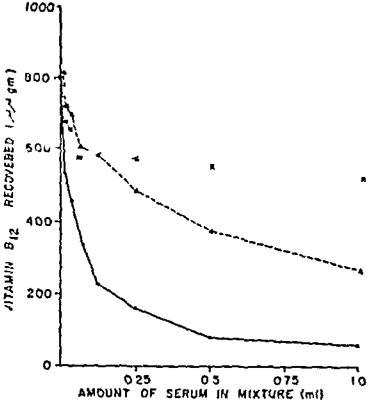

Fig 6

Fig 4 —Vitamin B_{12} binding capacity of 13 sera of patients with chronic granulocytic leukemia after effective treatment (▲--▲--▲) in comparison with vitamin B_{12} binding capacity of 25 sera of patients with chronic granulocytic leukemia before treatment (●——●) (Average curves and standard deviations)

Fig 5 —Vitamin B_{12} binding capacity of 11 sera of patients with lymphocytic leukemia (Average curves and standard deviations)

Fig 6 —Average curves of vitamin B_{12} binding capacity of serum obtained from normal individuals (▲--▲--▲), patients with chronic granulocytic leukemia (●——●), and lymphocytic leukemia (✕··✕··✕).

in whom the serum content of vitamin B_{12} varied from 140 to 860 $\mu\mu$g per milliliter for the total form and from 0 to 328 for the "free" form, were also studied with the method described above. The average curve and standard deviations of the results are shown in Fig. 5.

In addition, the serum binding capacity was studied in 3 cases of acute granulocytic leukemia, 3 cases of monocytic leukemia, and 6 cases of untreated pernicious anemia. The curves obtained with the serum from these patients were all within normal limits and are not reported here.

In Fig. 6, the average curves obtained in normal, lymphocytic leukemia and chronic granulocytic leukemia sera are shown together for comparison.

DISCUSSION

The simple vitamin B_{12} determinations in our series of patients showed results comparable with those obtained by others. Namely, there was an increased amount of total vitamin B_{12} in the serum of patients suffering from chronic granulocytic leukemia, while in serum of patients suffering from acute and chronic lymphocytic leukemias, the values of total vitamin B_{12} were within normal limits.

When pure vitamin B_{12} was added to the different types of sera, a striking difference in the recovery of the substance was observed. The vitamin added to normal sera never became entirely unavailable to the microorganism, while the substance added to sera obtained from patients suffering from chronic granulocytic leukemia was often completely undetectable by assays even at the very high dilutions of sera. The sera of patients suffering from lymphocytic leukemia, acute or chronic, bound less vitamin than chronic granulocytic leukemia and even less than normal serum. The levels of "free" vitamin B_{12} in acute and chronic lymphocytic leukemia were elevated in comparison with those of normal and of chronic granulocytic leukemia (Table I).

The high values of serum vitamin B_{12} in chronic granulocytic leukemia decreased progressively as the patient responded favorably, with lowering of the leukocyte levels, to treatment with either Myleran or x-ray therapy. When the number of white blood cells fell to normal values, the binding capacity of the sera also became normal. In Fig. 3, the index of binding capacity is arbitrarily represented as the amount of vitamin B_{12} recovered when 1 ml. of a solution containing 800 $\mu\mu$g of the substance was added to 1 ml. of serum diluted 1:8. It can be seen that the amounts of vitamin B_{12} bound were higher when the serum levels of the vitamin were high, namely, during relapse when the leukocyte count was more elevated. Comparison between the binding capacity curves obtained in patients before and after treatment shows a striking difference (Fig. 4). Reduction of the total serum vitamin B_{12} and of the binding capacity was not observed regularly in patients whose disease was advanced and becoming clinically refractory to any treatment even though there was a fall in the leukocyte count. This is in agreement with the finding of Mollin and Ross.[6]

These findings seem to support the hypothesis that the high levels of vitamin B_{12} in chronic granulocytic leukemia are in some way connected with the elevated number of leukocytes. The decreased serum vitamin B_{12} binding capacity and the increase of the "free" form in chronic lymphocytic leukemia suggest a relationship between the absence of granulocytes and diminished avidity of serum for vitamin B_{12}. The hypervitaminosis observed in cases of nonleukemic leukocytosis[1-6] is also in agreement with this concept. Were such the case, the vitamin should originate from the granulocytes or the substance should become bound, after intestinal absorption, to a protein liberated or produced by the leukocytes. This explanation seems to be the simplest one and if such a hypothesis is excluded, the interpretation of the phenomenon of hypervitaminosis is more difficult.

Nevertheless, many other findings cannot be explained by these hypotheses. Beard and his associates[3] observed a correlation between leukocytosis and levels of vitamin B_{12} in the serum of chronic granulocytic leukemia patients but this correlation was not evident in cases of acute leukemias.[4] Harris[9] showed that normal leukocytes contain significant amounts of vitamin B_{12} but Thomas and Anderson[10] showed that the amount of vitamin B_{12} in the leukocytes of patients suffering from leukemia is not increased. Mollin and Ross[6] were unable to demonstrate that the leukocytes were the source of vitamin B_{12}. They showed that the leukocytes could release material capable of binding vitamin B_{12}. Thomas and Anderson,[1] however, demonstrated that leukemic leukocytes do not differ from normal leukocytes in their vitamin B_{12} binding capacity. If the increased serum level and the increased binding capacity are connected with the presence of granulocytes, one would expect a decrease of the substance and of the binding capacity in cases of aplastic anemia or agranulocytosis. Neither of these phenomena was, however, observed by Beard et al.,[3] Mollin and Ross[6] or by ourselves.

In pernicious anemia the curves of the binding capacity were normal in spite of the lower vitamin B_{12} content of the serum and the leukopenia. We observed, moreover, that while the levels of vitamin B_{12} vary in general with the fluctuations of the leukocyte count in *a single patient*, the vitamin B_{12} content is not necessarily directly related to the number of granulocytes in circulation. In other words, if two subjects have a leukocyte count of 40 000, in one there may be a serum level of vitamin B_{12} of 2 000 $\mu\mu g$ per milliliter and in the other of 10 000 $\mu\mu g$ per milliliter. We have not observed serum levels higher than 20,000 $\mu\mu g$ per milliliter even with extreme leukocytosis.

Falling serum vitamin B_{12} levels during treatment of chronic granulocytic leukemia seems to parallel the decrease in leukocytes. Only occasionally a questionable transient rise of the serum vitamin B_{12} could be observed after initiation of effective treatment followed by the fall of the values. This phenomenon would perhaps be of greater magnitude if the vitamin B_{12} originated from the leukocytes. Perhaps the most convincing argument against the granulocytes as the source of serum vitamin B_{12} is the fact that hypervitaminosis is not limited to chronic granulocytic leukemia but is found also in cases of cirrhosis of the liver,[8, 11] infectious hepatitis,[11] and polycythemia vera[6] in which diseases elevation of the number of leukocytes is not an obligatory feature. The vitamin B_{12} binding capacity of serum in these diseases was not measured.

Rachmilewitz, Aronovitch, and Grossowicz[11] postulated that the increase of serum vitamin B_{12} in liver diseases is due to the release of the stored vitamin from damaged liver cells. While this is possible, the excess vitamin would probably be rapidly excreted unless there were greater retention. Such increased retention has been shown to be present in chronic granulocytic leukemia[7] and is presumably due to enhanced binding capacity of the plasma. Another interpretation of this phenomenon is that in certain diseases and especially in chronic granulocytic leukemia, the plasma contains an abnormal protein or an increased amount of a normal protein which binds vitamin B_{12}. By means of electrophoresis, it is possible to locate the zone where vitamin B_{12} is bound. This is found at the level of the alpha-1 and alpha-2 globulins, both in normal and in leukemic sera.[7] No qualitative differences could be demonstrated by this technique.

No differences in protein content can be detected between normal and leukemic sera by the usual chemical means. Nevertheless, the possibility of minimal quantitative differences cannot be excluded since the amounts of vitamin assayed are extremely small and the method used for the measurement of serum protein is, of course, much less sensitive than the microbiological method used for vitamin assays.[6] An increase of the globulins has been demonstrated in the serum of chronic granulocytic leukemia[12-14] with more refined techniques.

In conclusion, it seems that the phenomenon of hypervitaminosis B_{12} in chronic granulocytic leukemia can best be explained by assuming as is probably the case, that the cyanocobalamin is absorbed in normal amounts, but in the presence of increased quantities of a binding protein, apparently an alpha globulin, the vitamin remains in the plasma until high levels are attained. The source of this binding material is unknown. The hypothesis that the binding substance originates from the leukocytes deserves further investigation. It is possible that the method for the measurement of binding capacity described in the present paper can provide an indirect estimation of this vitamin B_{12} binding substance.

SUMMARY

A method for the measurement of binding capacity of serum for vitamin B_{12} is described. With this method, sera from normal subjects and patients suffering from chronic granulocytic leukemia, lymphocytic leukemia (acute or chronic), and various other conditions were studied. The serum of chronic granulocytic leukemia patients showed an increase and that of lymphocytic leukemia patients a decrease of the binding capacity for vitamin B_{12}.

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THE ARGINASE ACTIVITY OF ERYTHROCYTES AND LEUKOCYTES WITH PARTICULAR REFERENCE TO PERNICIOUS ANEMIA AND THALASSEMIA MAJOR

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ARGINASE has long been known to be present in various human tissues. This enzyme was first described in 1904,[1] and subsequently it has been identified in several human tissues including liver, breast, kidney, testes, salivary glands, epidermis, and erythrocytes. Its activity in liver has repeatedly been shown to be greater than that of other tissues and many times that of blood. Krebs and Henseleit[2] established the importance of hepatic arginase in the cycle by which amino nitrogen is converted into urea during the catabolism of amino acids. The physiologic significance of arginase in other tissues has never been clearly defined. Arginase has long been considered one of the most specific of enzymes. It has been found in both the cytoplasm and nuclei of cells, but as yet there is no satisfactory histochemical technique for its measurement. No attempt will be made to further summarize the existing knowledge about this enzyme since excellent reviews are available.[3]

Several investigators have concerned themselves with the arginase activity of human blood. Weil and Russell[4] were among the first to demonstrate its presence in human erythrocytes. Covolo and West[5] concluded that red cell arginase activity varied with each individual. The results of Clark and Beck[6] suggested that there was no significant variation in the activity of this enzyme with aging or pregnancy. Kochakian and his associates[7] measured the activity of erythrocyte arginase in various diseases and were the first to point out an increased value in pernicious anemia, either untreated or in relapse. They further demonstrated that the erythrocyte arginase activity decreased progressively as these patients showed hematologic improvement. They also suggested from their data that a diurnal variation in enzyme activity exists, that there is no relation of activity to meals, and that levels for a given individual were not constant when serial assays were performed over a year.

Although the existence of arginase has been denied in human leukocytes,[7] the purpose of the present study was to assay human leukocytes and erythrocytes for arginase in the normal as well as the abnormal individual, applying newer methods for the separation of formed element of blood.

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METHODS

Separation of erythrocytes from leukocytes was accomplished in the manner previously described.[8,11] A 4 per cent solution of bovine fibrinogen (Sigma) was found to provide optimal yields. The entire procedure was carried out in a cold room at environmental temperature of 4° C. After sedimentation of the red cells, the leukocyte-rich supernatant plasma was aspirated. Leukocytes were separated from the platelets and plasma by centrifugation at 1,000 r.p.m. for 10 minutes. Thereafter, the leukocytes were washed twice with normal saline. The preincubation suspension of white cells was adjusted so as to give a final cell concentration of 45,000 to 50,000 per cubic millimeter. This final suspension was then counted in quadruplicate using Bureau of Standards' certified red cell pipettes. An erythrocyte contamination of about 50,000 cells per cubic millimeter was considered insignificant in so far as no detectable arginase activity could be demonstrated on repeated arginase determination in this range of pure erythrocyte concentration.

The erythrocytes were processed in a slightly different way. After aspiration of the leukocytes, the remaining erythrocyte-plasma suspension was washed twice with saline at centrifugation speeds of 1,500 r.p.m. Platelet contamination was minimal. Moreover, assays of highly concentrated platelet suspensions for arginase had previously given negative results.[11] The erythrocyte suspension in saline was then adjusted by dilution to provide a concentration of 1.0 to 1.5 million cells per cubic millimeter so that final colorimeter readings would fall within a range of optical densities conforming to Beer's law. After quadruplicate cell counts had been taken, this suspension was centrifuged at 2,500 r.p.m. for 10 minutes, the supernatant saline was aspirated and discarded, and the original volume was restored with glass-distilled water. Neither homogenization of cells in distilled water nor addition of saponin appeared to increase the enzyme yield from white cells. Consequently the leukocytes were intact in saline suspension at the time of addition to the substrate. By way of contrast, enzyme activity of erythrocytes was negligible unless these cells were hemolyzed in distilled water prior to incubation. Addition of saponin failed to further increase erythrocyte arginase activity.

Arginase activity was determined by a modification of the methods of Kochakian[7] and Kingsley.[12] The following reagents were used:

1. L-arginine monohydrochloride, 0.047 M (1 per cent) solution in glass-distilled water, pH adjusted to 9.9.
2. Diethylbarbiturate buffer, pH adjusted to 9.9.
3. Cobaltous chloride, 0.0015 M solution in glass-distilled water.
4. Hydrochloric acid, 0.3 N.
5. Phosphate buffer (3 parts 0.2 M KH_2PO_4 to 7 parts 0.2 M Na_2HPO_4 solution). The pH must be adjusted to a point (ca. 12.0) so that addition of 1.0 ml. of this buffer to the arginase digest will lower the pH of the resultant mixture to 7.0.
6. Urease solution (1.0 Gm. of urease, 1.5 Gm. of Permutit, and 15 Gm. of sodium chloride are brought to a volume of 50 ml. with glass-distilled water, mixed by shaker for 30 minutes, allowed to stand at room temperature for 2 hours, filtered through Whatman No. 42 paper and adjusted to pH of 7.0).
7. Stock standard urea solution (400 mcg. of urea N per milliliter).
8. Acid cadmium sulfate solution (5.77 Gm. of $3CdSO_4 \cdot 8H_2O$ and 28.2 ml. of 1 N H_2SO_4 are brought to a volume of one liter with distilled water).
9. Sodium hydroxide 1.1 N.
10. Nessler's Reagent.

For each determination 2 unknowns, a blank and a standard were run. Each unknown incubation mixture consisted of 2.0 ml. of arginine solution, 1.0 ml. of barbiturate buffer, 0.25 ml. of cobaltous chloride solution, and 0.75 ml. of glass-distilled water. After equilibration in a water bath at 38° C., 1.0 ml. of cell suspension was added to each unknown. The blank contained the same constituents except for the addition of 1.0 ml. of 0.3 N HCl

Upon addition of the cells the blank tube was placed in a boiling water bath for 5 minutes in order to arrest all enzyme activity. The standard solution differed from the blank only by the substitution of 0.75 ml. of stock urea standard for glass-distilled water.

After addition of the white cells or the laked red cells, each solution was thoroughly mixed and incubated in a 38° C. water bath for 6 hours. Mixing at half-hour intervals was necessary to prevent settling of the protein coagulum. Each test tube was covered with a spherical marble to minimize diminution of volume by vaporization. At the end of 6 hours the arginase was inactivated by adding 1.0 ml. of 0.3 N HCl, mixing, and immersing the unknown test tubes in a boiling water bath for 5 minutes. Sufficient time was allowed for cooling before the addition of 1.0 ml. of phosphate buffer. A heavy brown precipitate was then formed by thoroughly mixing and was discarded after centrifugation at 2,500 r.p.m. for 10 minutes. The resulting supernatant could be saved for at least 3 days in a cold room or refrigerator before exposure to the action of urease.

In order to measure the urea so formed, 3 drops of urease solution were mixed with a 0.5 ml. aliquot of each unknown, blank, and standard supernatant, preheated in a water bath at 38° C. After frequent shaking throughout, this incubation was terminated at the end of 30 minutes by addition of 8.5 ml. of acid cadmium sulfate solution. Calibrated syringe pipettes were found useful for this purpose. After one minute 1.0 ml. of 1.1 N NaOH was added. The resultant mixture was shaken thoroughly to enhance flocculation and then allowed to stand 10 minutes before centrifugation at 2,500 r.p.m. for 10 minutes.

Final determinations were made in a Beckman Model-B spectrophotometer, using a blue lamp and wave length of 540 mμ. A 2.0 ml. aliquot of each clear supernatant was pipetted into spectrophotometer tubes. Nine milliliters of distilled water was then added by calibrated syringe pipette to each tube and carefully mixed. One milliliter of Nessler's reagent was added, and the optical density of the resultant amber solution read within one minute to avoid spurious readings caused by fine colloidal turbidity.

The values for arginase activity were expressed as milligrams of urea nitrogen per 10^{10} W.B.C. or per 10^{11} R.B.C. liberated in one hour from arginine substrate at 37° C. The figure for erythrocytes was chosen in order to avoid decimals below unity.

RESULTS

The erythrocyte and leukocyte unit cell arginase values are presented in the form of scattergrams (Figs. 1 and 3) in order to reflect the differences among several disease groups.

Erythrocytes.—Normal subjects: These were comprised of patients hospitalized for elective surgical conditions such as herniae and for chronic nonhematologic disorders such as arteriosclerotic brain disease. These patients were studied before surgery and all were in apparent good health apart from some nonsystemic disease. All patients were adults, and the majority were men. No attempt was made to analyze the results with regard to sex or age. Subjects were considered "normal" only if their hemoglobin, white blood count, and differential white blood count were within normal limits. Twenty-four such subjects were studied and values ranging from 0 to 46.2 mg. per 10^{11} R.B.C. were observed. The mean value was 21.0. In several instances repeat determinations were found to agree closely with the original value. All but 3 of the values fell within a range of 9.0 to 36.0, although it is quite probable that an increased number of normal subjects would show a somewhat greater spread. The normal mean erythrocyte arginase activity per unit cell was found to be about one-fiftieth that of normal leukocytes.

Pernicious and other nutritional macrocytic anemias: Sixteen patients with megaloblastic anemia (Table I) were studied while in hematologic relapse

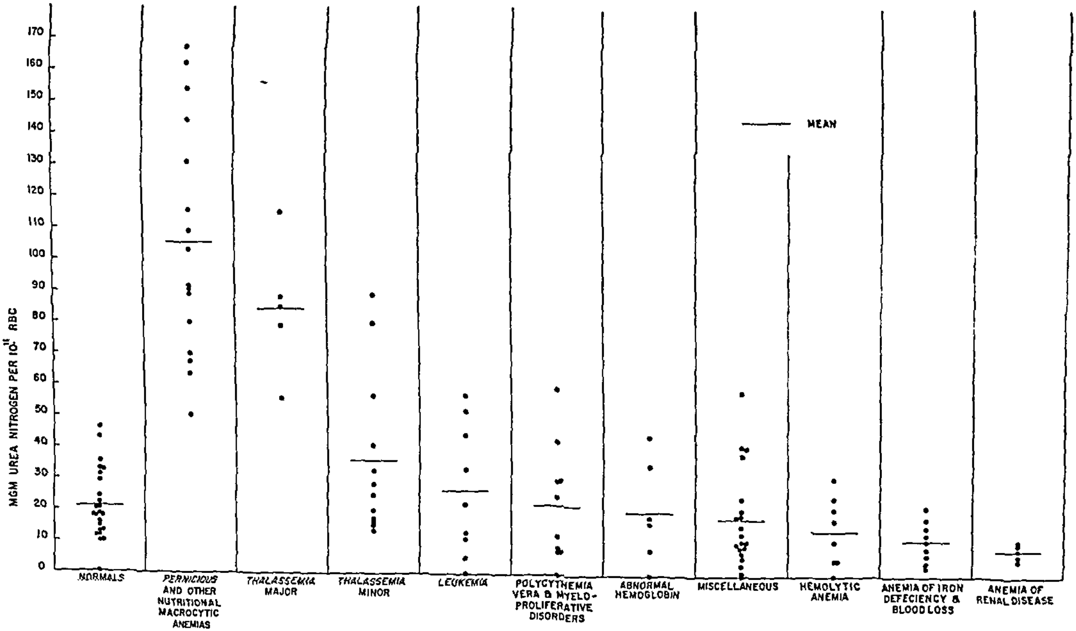

Fig 1—Scattergram of arginase activity of erythrocytes obtained from normal human subjects and subjects with various disease states Each dot represents a different patient Fourteen patients had typical Addisonian pernicious anemia in relapse while the other two in the same column had a vitamin B_{12} responsive megaloblastic anemia associated with regional enteritis Values for arginase activity are expressed as milligrams of urea nitrogen liberated *per 10^{11} erythrocytes*

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Pernicious and other nutritional macrocytic anemias: Sixteen patients with megaloblastic anemia (Table I) were studied while in hematologic relapse or at the time of initiation of appropriate treatment. Fourteen of these were considered to exhibit typical Addisonian pernicious anemia by virtue of histamine anacidity, a bone marrow showing typical megaloblasts, a distinct reticulocyte response to either B_{12} or folic acid therapy, and in several instances *characteristic fecal or urinary excretion patterns of* radioactive cobalt, as described in the methods of Heinle[13] and Schilling.[14] Two patients suffered from regional enteritis complicated by multiple surgical procedures. Both showed free hydrochloric acid at the time of gastric analysis and both responded well hematologically to parenteral administration of vitamin B_{12}.

The values for erythrocyte arginase of these 16 patients ranged from 50.2 to 167 with a mean of 105.4. As can be seen from the scattergram all values are well in excess of that of the highest normal subject. The mean of the untreated megaloblastic anemias is more than 5 times that of normal subjects.

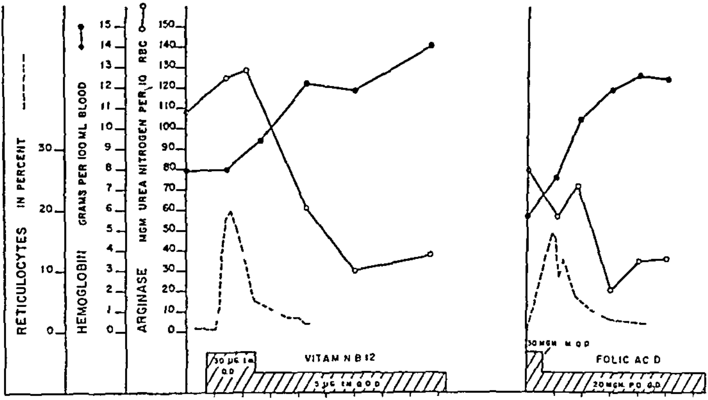

Fig. 2.—Progressive lowering of erythrocyte arginase activity in two different patients with pernicious anemia. One received only vitamin B_{12} and the other received only folic acid during the period of study. Each point represents a separate arginase, hemoglobin and reticulocyte determination.

Eight of these patients were followed serially over periods up to 90 days as they were receiving treatment. One patient, who showed no signs of subacute combined system disease, was purposely placed on folic acid therapy for *a period of one month in an attempt to compare his arginase response to that* of patients receiving vitamin B_{12} therapeutically. He developed a typical reticulocyte and hemoglobin response while on folic acid (Fig. 2) as well as a progressive diminution of erythrocyte arginase activity. In all instances of serial determinations a progressive diminution of the arginase value occurred *beginning between the second and fourth week of therapy*. The reticulocyte, hemoglobin, and arginase response to vitamin B_{12} therapy of another patient with typical pernicious anemia in relapse is shown in Fig. 2. This response typifies that of the other patients studied serially in this fashion. The first posttreatment arginase value was higher than the pretreatment level in 2 other patients who were followed serially.

Four cases with both subacute combined system disease and Addisonian pernicious anemia proved in each instance by histamine anacidity, megaloblastic bone marrow, and appropriate hematologic response to vitamin B_{12} were studied. Each had received continuous hematinic therapy for periods ranging from 2 to 6 years. All 4 patients yielded values (18.2, 11.2, 9.1, and 4.2) comparable to those of normal subjects.

Thalassemia: Five cases of thalassemia major showed a range of 55.7 to 114.9 with a mean of 84.6. All 5 of these determinations were obtained through the cooperation of Dr. Phillip Sturgeon. The essential data on these 2 groups of siblings is shown in Table II. It is of interest that fetal hemoglobin determinations were relatively higher in the first 3 patients than in the last 2, although all 5 were distinctly elevated. The parents of both groups of children had been previously studied and shown to possess a microcytic hypochromic blood picture consistent with thalassemia minor.

TABLE II. ERYTHROCYTE ARGINASE IN THALASSEMIA MAJOR

PATIENT	HEMOGLOBIN (GM. PER 100 ML.)	R.B.C. ARGINASE (MG. UREA N/10^{11} R.B.C.)
Es. C.	10.6	87.9
Ed. C.	9.4	85.3
T. C.	9.4	55.7
Ch. L.	8.8	114.9
Co. L.	9.2	79.2

Twelve patients with thalassemia minor were included in this study. In all instances the red cell morphology by Wright-stained smears appeared hypochromic and microcytic. The majority of this group were of Mediterranean origin, and in several instances evidence of similar findings in other members of their immediate families was available. Red cell indices in all cases confirmed the hypochromic microcytosis, although the mean corpuscular hemoglobin concentration (M.C.H.C.) values were generally considerably higher relative to the mean corpuscular hemoglobin (M.C.H.) value. It was felt that in this respect the pattern of their indices was in distinct contrast to that of chronic iron deficiency anemia in which greater depression of the M.C.H.C. is usually seen. As shown in the scattergram, a considerable range exists between the maximum and minimum erythrocyte arginase values for patients with this condition. The lowest value was 13.5, and the highest was 89.2 with a mean of 36.3. Most values were therefore within the normal range, though the mean is somewhat high.

Iron deficiency anemia: Nine individuals with histories of recent or chronic blood loss or with anemias responding promptly to therapeutic iron were studied. The arginase values under these circumstances range from 2.7 to 22.0 with a mean of 11.5. The scattergram emphasizes that there is less spread and a lower mean value when this group is compared with that of thalassemia minor One patient was a 36 year old woman with unexplained melena who presented with a hemoglobin of [illegible]9 Gm per 100 ml Her initial smear showed marked hypochromia and microcytosis, and the erythrocyte indices were calculated as M C V, 71, M C H 18 and M C H C, 26 The patient's bleeding ceased spontaneously, and in the absence of transfusions her hemoglobin returned to a value of 13 1 Gm with a maximum reticulocytosis of 7 9 per cent on oral iron therapy over a period of 6 months Her arginase value was 4 3 An 8 month old male infant without evidence of blood loss but with an unequivocal dietary history of marked iron deficiency was studied when his hemoglobin value was 4 2 Gm per 100 ml Erythrocytes appeared markedly hypochromic and microcytic on smear His erythrocyte arginase was 11 5 Five patients exhibited iron deficiency anemia as a result of gastrointestinal bleeding from duodenal ulcers multiple telangiectases, and hemorrhoids Their arginase values were 2 7, 8 8, 13 2, 18 5 and 22 One woman with long standing hypoparathyroidism accompanied by an iron deficiency anemia showed an erythrocyte arginase activity of 15 5 In a child with a patent ductus arteriosus, whose serum iron was 16 5 μg an arginase value of 6 8 was obtained

Anemia of azotemia In view of the findings of Liener and Schultze[15] that excessive liver arginase activity in uremic newborn rats could be reversed by the addition of vitamin B_{12} in vivo, examination of patients with uremia and/or chronic renal disease seemed pertinent Five such patients were studied and showed arginase values ranging 5 3 to 11 3 with a mean of 8 5, as shown in the scattergram The clinical diagnoses in this group included chronic pyelonephritis, chronic glomerulonephritis, arteriolar nephrosclerosis (2 patients), and malignant nephrosclerosis All 5 patients were moderately anemic and all showed nitrogen retention Their blood urea nitrogen values ranged between 31 and 168 mg per 100 ml The arginase activity of erythrocytes in patients with azotemia correlated with neither the severity of anemia nor degree of nitrogen retention

Hemolytic anemia Eight patients with various frank hemolytic anemias were studied There were two cases of idiopathic acquired hemolytic anemia both of whom had previously been splenectomized, one of congenital hemolytic jaundice, two of paroxysmal nocturnal hemoglobinuria, one of nonspherocytic hemolytic anemia and porphobilinogenuria, one of a severe hemolytic anemia secondary to Hodgkin's disease, and one of a hemolytic anemia associated with myelofibrosis and myeloid metaplasia Some of these patients had in the past required transfusions, but in no case had blood been given within a month of arginase determination The possibility that a small proportion of the red cells sampled were foreign to the recipient must still be considered The scattergram depicts the range of 0 to 30 9 found in the hemolytic anemias and the mean of 14 4 It is of note that one of the patients with idiopathic acquired hemolytic anemia, whose arginase value was 30 9, showed a reticulocytosis of 38 per cent The patient with myelofibrosis and myeloid metaplasia, whose arginase was 11, was of unusual interest in so far as his differential count revealed 396 nucleated erythrocytes per 200 white blood cells. It should not be inferred that the arginase activity of nucleated red cells is wholly included in the measurement of erythrocyte arginase, since the majority of the former cells are probably removed at the time of separation of the leukocyte plasma phase.

Leukemia: The erythrocyte arginase values of 9 cases of leukemia, all of which were anemic, are shown on the scattergram. One case of acute blastic leukemia with an arginase value of 12.9 is included. Three cases of chronic lymphatic leukemia were found to have arginase values of 11, 4.9, and 33.3. The 5 cases of chronic myelocytic leukemia were selected on the basis of peripheral smears, bone marrow picture, low leukocyte alkaline phosphatase value,[17] clinical course, and autopsy findings. Values for erythrocyte arginase of 57.1, 52.1, 44.3, 22.2, and 0 were found in this group of patients. Three of these values for chronic myelocytic leukemia are above the mean of normal subjects.

Polycythemia vera and myeloproliferative disorders: Polycythemia vera and certain myeloproliferative disorders not considered to be either polycythemia or leukemia are arbitrarily grouped together as a matter of convenience. Four cases were considered typical of polycythemia vera and showed arginase values of 59.1, 12.3, 8.7, and 7.1. The highest of these is noteworthy in that he exhibited the anemic and leukemoid phase of his disease at the time of the arginase determination, whereas 2 years previously he demonstrated the classical features of polycythemia vera and had, therefore, received internal irradiation with P^{32}. The other 6 patients include 3 of idiopathic myeloid metaplasia, [illegible]f myelofibrosis with myeloid metaplasia of the spleen, and one of osteosclerosis, [illegible]embling Albers-Schonberg disease. All 6 of these patients were moderately anemic. Their erythrocyte arginase values were 42.6, 29.9, 7.3, 24.9, 0, and 30, respectively. The erythrocyte arginase activities of this arbitrary group are shown in the scattergram.

Abnormal hemoglobins: Five patients with abnormal hemoglobins were studied. In addition one specimen of cord blood (ordinarily high in hemoglobin-F content) was obtained at the time of normal delivery. The hemoglobinopathies included 2 patients with sickle-cell trait (S-A), one with classical sickle-cell anemia (S-S), one with sickle-cell hemoglobin-C disease (S-C), and one with sickle-cell hemoglobin-D disease (S-D). The last mentioned patient has been reported separately by Sturgeon and co-workers.[17] His arginase value was 44.2, while those for the cord blood and the patients with sickle-cell trait, hemoglobin-S-C and homozygous-S, were 18.5, 1.8, 0, 34.5, and 16.6, respectively. These values can be seen on the scattergram.

Miscellaneous: The erythrocyte arginase values of 20 patients with various unrelated disorders are shown in the scattergram. The majority of these patients were mildly to moderately anemic. The highest value in the miscellaneous group (56.8) was obtained from a patient with Laennec's cirrhosis, jaundice, ascites, and anemia. Three cases of diabetes mellitus and 5 anemias of infection had normal arginase values. Other diseases in which normal values for erythrocyte arginase were obtained include multiple myeloma, 2 leukemoid reactions, 4 cases of primary refractory anemia, acute intermittent porphyria, and metastatic hypernephroma. One patient in the miscellaneous group was selected for study because of a failure to absorb an oral tracer dose of Co60 labeled vitamin B_{12} probably conditioned by total gastrectomy 2 years earlier. Although this patient had not received vitamin B_{12} or folic acid prophylactically, no signs of anemia had developed. Moreover, his arginase value of 38.2 is comparable to that of normal subjects and falls below the minimum among patients with untreated megaloblastic anemia.

Leukocytes.—The results of leukocyte arginase determinations in various disorders are represented by another scattergram (Fig. 3). Normal subjects were selected in the same manner as for erythrocyte determinations. Leukocyte arginase values were expressed in terms of milligrams of urea nitrogen per 10^{10} W.B.C. The mean of 22 normal individuals was 103.4. The number of cases of leukemoid reactions were too few to justify the conclusion that the mean of 237 represented a significant increase over the normal. The scattergram emphasizes the wide spread of arginase values in normals, chronic myelocytic leukemia, polycythemia vera and other myeloproliferative disorders, infections, and other leukocytoses. No significant differences in leukocyte arginase activity among these disorders could be found. In addition one case of each of the following diseases failed to disclose a significant deviation from the normal: thalassemia minor, rheumatoid arthritis, infectious mononucleosis without lymphocytosis, Hodgkin's disease, and pernicious anemia. Two other cases of pernicious anemia revealed leukocyte arginase activities of 289 and

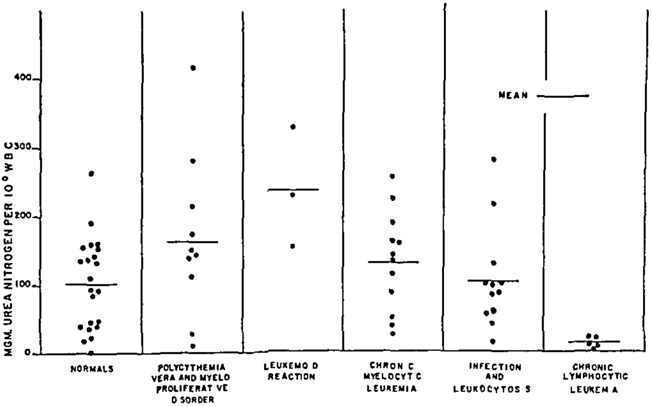

Fig. 3.—Scattergram of arginase activity of leukocytes obtained from normal human subjects and subjects with various disease states. Each dot represents a different patient. Values for arginase activity are expressed as milligrams of urea nitrogen liberated per 10^{10} leukocytes.

374. The very low values in all 5 cases of chronic lymphocytic leukemia are in distinct contrast to the other diseases plotted in the scattergram. In addition, 2 patients with acute blastic leukemia showed negligible leukocyte arginase activity. With the exception of these last 2 diseases, no consistent aberration from normal was observed in any of the disorders studied.

DISCUSSION

Whereas investigation of erythrocyte arginase activity showed significantly higher values in pernicious anemia, nutritional macrocytic anemias, and thalassemia major than in normal individuals and other types of anemia, no such difference was found for leukocyte arginase activity in various disorders. The most striking finding was the confirmation of the work of Kochakian and his co-workers,[7] in which a uniformly elevated erythrocyte arginase activity was found in pernicious anemia in relapse. We were able to add to this the impression that in other untreated nutritional macrocytic anemias some or all of the red cell population possesses increased arginase activity. In 2 patients with nutritional macrocytic anemia associated with regional enteritis this was found to be true. In these cases, however, there appeared to be a vitamin B_{12} deficiency, as is true in Addisonian pernicious anemia. Kochakian's work was further confirmed in so far as the initially high erythrocyte arginase activity returned to normal gradually as vitamin B_{12} administration induced hematologic improvement. In one patient the same sequence of gradual lowering of arginase activity during the first month was noted when folic acid was used instead of vitamin B_{12}. The effects of citrovorum factor were not investigated, although it is predicted that similar results would have also occurred with administration of this substance. The exact time of reversal of the elevated arginase activity toward normal after the initiation of vitamin B_{12} or folic acid is difficult to predict. This generally occurs between the second and fourth weeks. Some patients still showed slightly increased enzyme activity after one month of therapy. It is likely that these patients at the end of 6 months would have yielded normal values. Four cases of proved megaloblastic anemia, all treated satisfactorily for periods well in excess of one year, showed normal erythrocyte arginase activity. Disproportionate amounts of unit cell arginase in the total red cell population of an individual may explain the variability in the time required to restore the arginase value to normal. Erythrocytes formed during a deficiency of vitamin B_{12} appear to contain excessive amounts of arginase. Since the destruction and cessation of bone marrow release of such cells are not necessarily constant among different individuals with pernicious anemia receiving treatment with vitamin B_{12}, the amount of arginase activity after a constant time interval also varied with the patient. In 2 of the 7 patients that were followed serially, the erythrocyte arginase activity of the second specimen, drawn one week after onset of vitamin B_{12} therapy, exceeded the initial base-line value by 31 and 16 per cent. A similar phenomenon was not necessarily excluded in the other patients in so far as successive arginase determinations were relatively infrequent in most of the patients studied. It can only be suggested that this increase in enzyme activity soon after the onset of therapy may have been related to a more rapid bone marrow release of abnormal cells which had undergone most of their medullary development prior to vitamin B_{12} therapy.

A positive correlation between the intensity of erythrocyte arginase activity and severity of anemia in terms of hemoglobin of venous blood is apparent only in cases of megaloblastic anemia. The highest value thus far encountered was noted in a patient whose hemoglobin was 2.7 Gm. per 100 ml. at the time of admission for megaloblastic anemia following resective and short circuiting surgery for regional enteritis. Use of a suspension count of 500,000 R.B.C. per cubic millimeter rather than the customary one of about 1,000,000 provided an even higher final unit cell activity of 206 in this particular case. The erythrocyte arginase activity of the rest of the cases of pernicious and other macrocytic anemias tended to be higher when the degree of anemia was more severe. The most anemic of the 5 patients with thalassemia major in terms of hemoglobin provided the highest arginase value in this small group. Such a correlation could easily be due to chance. Certainly no consistent relationship between hemoglobin and arginase is evident in the other conditions studied.

Since it is not known whether the arginase present in red cells bears a relationship to stroma, hemoglobin, or some other moiety, final expression of enzyme activity in terms of a unit cell was elected. We recognize that the size of erythrocytes in pernicious anemia usually considerably exceeds that of the normal. This difference in size, however, could in no way account for the elevated arginase activity of patients with nutritional macrocytic anemias relative to that of normal individuals. In fact, the observations of Kochakian and his associates[7] are in close agreement with ours with respect to high erythrocyte arginase activity in pernicious anemia, despite several differences in method. Among these was the addition of a given volume of erythrocytes to the incubation mixture rather than of a given number of cells, as done in the present study.

The findings in Cooley's anemia were almost as striking as those in pernicious anemia. In both conditions a consistently marked elevation of arginase activity of red cells occurred in comparison to a group of normal patients and to several other anemias. The finding of elevated levels of this enzyme in the erythrocytes of 5 out of 5 cases of thalassemia major does not allow one to conclude that such an observation is true in every patient with this disease. Many additional determinations would be needed to settle this point. Unfortunately, the rarity of Cooley's anemia in the Los Angeles area is a hindrance to statistically significant figures. In contrast to thalassemia major, erythrocytes of patients with thalassemia minor showed a variable pattern of arginase activity, ranging from normal to a few distinctly elevated values. Because of the overlap erythrocyte arginase determinations as a clinical tool would not appear to offer any value in distinguishing between a severe thalassemia minor and very mild thalassemia major.

No explanation can be offered as to the reason for high arginase values in pernicious anemia and thalassemia major nor for the similarly high values in these two particular forms of severe anemia. A deficiency of vitamin B_{12} and related substances has never been demonstrated in thalassemia. There is a striking similarity in erythrocyte morphology and in resistance to iron therapy between thalassemia and the experimental anemia in pyridoxine-deficient animals.[18] Vitamin B_6, however, has been administered to patients with thalassemia major without hematologic benefit.[19] Experimental pyridoxine-deficiency anemia in humans has been produced only with great difficulty.[20] More recently a well-documented instance of severe anemia responding to parenteral vitamin B_6 has been reported.[21] Certainly a relationship between the arginine-ornithine-urea cycle and pyridoxine has been suggested in the work of McHenry and his collaborators.[22] They have repeatedly found that the fasting blood urea level is abnormally high in vitamin B_6-deficient rats. Of equal significance is the observation of Liener and Schultze,[15] who found that vitamin B_{12} seemed to bear a similar relation to urea formation. The offspring of rats reared on a diet of crude plant material were uremic at birth but could be cured by the addition of vitamin B_{12}. It has been suggested that both of these observations support a view that both vitamin B_{12} and vitamin B_6 enhance the utilization of circulating amino acids for protein synthesis. Of particular interest is the observation that Liener's uremic rats also demonstrated distinctly elevated hepatic arginase values before they received vitamin B_{12}.[15] However, while both vitamin B_{12} and pyridoxine deficiency appear in some experimental situations to result in increased blood urea values and while arginase is an important enzyme in the metabolism leading to urea formation, no reason for high arginase values in these two types of anemia is clearly present. *While certain similarities exist between thalassemia and pyridoxine deficiency anemia, it should be emphasized that no relation of pyridoxine to the defect in thalassemia has ever been demonstrated.* It is of interest, however, that of the wide variety of anemic states studied for arginase activity, only anemias due to vitamin B_{12} deficiency and the anemias of thalassemia show the marked departure from normal.

In view of the morphologic similarity between the erythrocytes of thalassemia and those of iron deficiency states, it was considered important to assay arginase activity in both of these groups. The normal or possibly low values of iron deficiency are in contrast to the elevated levels of thalassemia major and the sometimes elevated levels of thalassemia minor. The complexity of the method for determining arginase does not justify proposing its use as a tool in the differential diagnosis of thalassemia and iron deficiency.

Although it was not possible to study any patients exhibiting hemoglobinopathies involving combinations of other abnormal hemoglobins with the thalassemia gene, erythrocyte arginase determinations were done on cord blood, homozygous sickle-cell, sickle-cell hemoglobin-C, and sickle-cell hemoglobin-D bloods. The only hemoglobinopathy showing an elevated arginase value was sickle-cell hemoglobin-D disease, and this was only very moderate. The rarity of this disorder would make further confirmation of this finding in other patients difficult. Because of the similarity of morphology between hemoglobin-H disease, thalassemia minor and iron deficiency anemia, erythrocyte arginase assays in the former disorder would be of interest. The original description of hemoglobin-H disease characterizes this condition as a hypochromic microcytic anemia refractory to iron therapy, with reticulocytosis, splenomegaly, decreased osmotic fragility to hypotonic saline and diminished red cell survival.

Other conditions in which erythrocyte arginase was found to be slightly higher than normal were chronic myelocytic leukemia and the anemia of chronic liver disease. Three of the 5 patients with chronic myelocytic leukemia that were studied showed somewhat elevated values. None of these patients were under treatment with antifolic acid agents. An explanation for this observation cannot therefore be readily made. It is possible that a defect in either folic acid or vitamin B_{12} metabolism existed in the patient with anemia of liver disease. Only one other similar case was studied and in this particular instance the erythrocyte arginase value was normal.

The results of leukocyte arginase assays in various hematologic disorders were much less remarkable than those of erythrocyte arginase. It would appear from our data that both normal and diseased leukocytes possess definite arginase activity. In no particular disorder did leukocyte arginase appear markedly or consistently elevated above that of normal controls. In chronic lymphatic leukemia, infectious mononucleosis, acute leukemia and the terminal blastic phase of chronic myelocytic leukemia, leukocyte arginase values were lower than normal. From these findings it can reasonably be inferred that myeloid as opposed to mononuclear or blast cells *contribute primarily to the total* arginase activity of leukocytes.

SUMMARY AND CONCLUSIONS

The arginase activity of erythrocytes and leukocytes in normal and diseased humans was assayed by a method employing L (+) arginase as the substrate and pure urease to convert urea to ammonium carbonate so that the latter might be measured spectrophotometrically after nesslerization.

Both erythrocytes and leukocytes possess arginase activity, the latter possessing 50 times as much activity per mean unit cell as the former. A considerable spread in the values for both cells was found in the normal subjects that were studied.

Erythrocyte arginase studies were of greater significance than leukocyte studies. Distinctly elevated erythrocyte arginase values were consistently found in patients exhibiting pernicious and other nutritional macrocytic anemias in relapse and thalassemia major. A similar consistent elevation was not observed in anemias of iron deficiency, azotemia, leukemia, inherited hemoglobinopathies, malignancies, infection, and of several other diseases. This method because of its complexity is not recommended in preference to other existing laboratory procedures for *establishing a diagnosis of pernicious anemia or* thalassemia major.

No distinctive pattern of leukocyte arginase values in various diseases affecting white cells was noted. When the major portion of the differential white count was replaced by nonneutrophilic leukocytes, arginase activity was lower than normal.

The role of arginase in blood corpuscles remains unknown. Its possible relationship to pyridoxine and cyanocobalamin is discussed.

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THE OXYGEN SATURATION OF BLOOD IN THE VENAE CAVAE, RIGHT HEART CHAMBERS, AND PULMONARY VESSELS OF HEALTHY SUBJECTS

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FOR the detection and localization of arteriovenous shunts within the heart and associated great vessels accurate definition of the degree of oxygen saturation of venous blood within the right chambers of the heart and great vessels in healthy subjects is essential. Previous investigators[1-4] have pointed out the marked variability in the oxygen content of blood withdrawn from the right atrium and to a less extent the right ventricle. This has been attributed to (1) variation in the subject's physiologic state with a resultant change in cardiac output in the interval between the collection of blood samples, and (2) laminar flow within the right heart chambers resulting in incomplete mixing of streams of blood of different oxygen saturations from the superior and inferior venae cavae and the coronary sinus.

Because of this variability in oxygen saturation of venous blood within the normal heart, a method of withdrawing repeated blood samples in rapid succession through a cuvette oximeter, an instrument of high relative accuracy,[5, 6] has been developed for use during cardiac catheterization in an attempt to reduce the effects of incomplete mixing and changing cardiac output to a minimum.[7, 8] This presentation is an analysis of the data on oxygen saturation of blood obtained in this manner in an adequate series of unanesthetized, ambulatory, healthy, adult subjects. In addition to defining the mean and ranges of oxygen saturation within the right chambers of the heart and great vessels and the differences in the oxygen saturation of blood from these various sites it has been possible to make some inference as to the relative importance of changes in cardiac output and laminar flow (incomplete mixing) on the variability of the values obtained at these various sites.

The data on oxygen saturation of blood herein reported were obtained during cardiac catheterization procedures, which also included recording of pressure, the inhalation of 95 per cent oxygen and, in some subjects, a period of exercise, the results of which are reported elsewhere.[9, 10] The subject numbers used in this and related publications are identical.

METHOD

Twenty-six healthy subjects have been included in this study. Data are arranged according to age of the subjects in Table I. Fifteen of these subjects were healthy male

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physicians and 11 were patients in whom cardiac catheterization was performed to exclude cardiac abnormalities. Subjects 7, 24, and 26 were women, the remainder men. A detailed description of the technique of cardiac catheterization used has been reported elsewhere.[9, 11] The subjects had a light breakfast and received codeine, 0.5 gr. (0.032 Gm.), and seconal, 1.5 gr. (0.1 Gm.), 15 to 30 minutes before the procedure commenced. The cardiac catheter was connected to both a cuvette oximeter for whole blood and to a strain-gauge manometer by means of a 3-way stopcock.[12] This assembly was kept filled with heparinized saline solution. Samples of blood were obtained from the catheter by applying manual suction to a syringe connected to the cuvette oximeter. Two or more samples were obtained in rapid succession in this manner through the catheter-cuvette system; following this the system was flushed with heparinized saline solution to prevent clotting. The position of the tip of the catheter was rapidly changed under fluoroscopic control between each sample. Spot roentgenograms were taken in many cases for later confirmation of the position of the tip of the catheter. Pressure pulses were also continuously monitored.

Samples of blood obtained in rapid succession without the introduction of saline solution between individual samples were separated in time by a maximum of 1 minute and have been called "consecutive." Samples which were separated by the introduction of saline solution into the system have been called "random" being separated in time by a minimum of 2 minutes. An attempt was made, particularly in the 11 patients to repeat each consecutive series of samples at least once, and when doubt arose as to the interpretation of the saturations obtained, 2 or more times. In the majority of the subjects the blood withdrawn through the cuvette oximeter was discarded. In some, however, the blood was reinfused through the catheter-cuvette assembly immediately after withdrawal of each series of samples was completed.

The oxygen saturation of arterial blood was recorded both continuously by oximeters attached to one or both ears,[13] and intermittently from samples withdrawn through an additional cuvette oximeter attached to a needle indwelling in the right radial artery.

The galvanometer deflections produced by the cuvette and ear oximeters were observed visually by the use of the single scale circuit and both single and double scale deflections were continuously recorded photokymographically.[14, 15] Subsequently a calibration curve was constructed for each subject which related the single scale galvanometer deflection to the oxygen saturation as obtained by Van Slyke[5] analysis.

Samples of blood were withdrawn simultaneously from the radial and pulmonary arteries with the subject breathing air and also when breathing 95 per cent oxygen and analyzed for oxygen content within 30 minutes by the method of Van Slyke and Neill.[16] The oxygen capacity was determined after equilibration by the method of Sendroy[17] as modified by Roughton and associates.[18] In addition, in most subjects a sample was obtained for analysis from the site of lowest oxygen saturation to assist in the calibration of the cuvette oximeters.

Ninety-five per cent oxygen was administered by a closed circuit spirometer system during the latter part of the procedure on all subjects, except Subject 25, for an average period of 78 minutes. Dye-dilution curves were recorded photokymographically by the earpiece oximeters and by the cuvette oximeter attached to the needle in the radial artery[19] following the injection of Evans blue dye at multiple central sites[20] soon after the subject began breathing oxygen. Because of the apparent depression in the oxygen saturation of blood (as recorded oximetrically) which was produced by the circulating dye, the absolute values for oxygen saturation with the subject breathing oxygen were not suitable for analysis. The differences in oxygen saturation between consecutive samples of blood obtained under these conditions were not disturbed and, therefore, have been included.

RESULTS

The oxygen capacities and oxygen content of blood determined by analysis of samples simultaneously withdrawn from the radial and pulmonary artery are given in Table I for each subject both while breathing air and while breathing oxygen. Other vital statistics including metabolic rate, cardiac output, and intracardiac pressures are reported elsewhere. The average cardiac index for the group was 3.5 (2.8 to 6.3) L./min./M.

The oxygen capacity was normal in all subjects averaging 19.7 vol. per cent with the subject breathing air with a range of 17.7 to 21.6 vol. per cent.[21] The average capacity of 19.6 vol. per cent while the subjects were breathing oxygen did not differ significantly from that while they were breathing air ($p < 0.1$). The mean total oxygen content of the systemic (radial) arterial blood (with the subject breathing air) was 19.4 (17.4 to 21.7) vol. per cent, and of the mixed venous (pulmonary artery) blood 15.3 (12.1 to 17.2) vol. per cent. While the subjects were breathing 95 per cent oxygen, these values were 21.2 (18.9 to 23.6) vol. per cent and 17.1 (13.4 to 19.4) vol. per cent, respectively. The arterial-venous difference in oxygen content of blood while the subjects were breathing air averaged 4.1 vol. per cent and did not change significantly during inhalation of 95 per cent oxygen.[9] The content of physically dissolved oxygen within the arterial blood (oxygen content minus oxygen capacity) during inhalation of 95 per cent oxygen averaged 1.6 vol. per cent and ranged from 1.0 to 2.6 vol. per cent.

The oxygen saturations obtained by cuvette oximeter of arterial (radial artery) blood and of venous blood withdrawn from the right heart chambers and great vessels are also given in Table I together with the saturations of the samples from the pulmonary artery wedge. The cuvette oximeter values given in Table I are the averages of the several samples withdrawn from each site in individual subjects while breathing air, the means and standard deviations being calculated from these values. However, the ranges given in the text and table are based on all the individual samples obtained. The mean and range of the oxygen saturation of arterial blood equalled 97.3 (95 to 99) per cent.

Samples of blood from the inferior vena cava were withdrawn with the catheter tip at or just above the level of the diaphragm, the catheter having been first advanced to below the diaphragm to confirm its position in the inferior vena cava and then withdrawn slightly. Samples from this position, obtained in 19 subjects showed a mean and range of oxygen saturation of 83.0 (76 to 88) per cent. The mean and range of the oxygen saturation of superior vena caval blood when samples were withdrawn with the catheter tip 1 to 4 cm. above the right border of the cardiac silhouette as seen fluoroscopically were 76.8 (66 to 84) per cent, a figure significantly less than the inferior vena caval saturation.

The catheter position at the time of withdrawal of right atrial blood varied considerably. In most cases it lay midway along the right cardiac border either near the tricuspid valve or adjacent to the lateral wall of the atrium. An analysis of the differences in saturation of blood obtained from various sites within the atrium was not attempted. Occasional blood samples of very low oxygen saturation withdrawn from the right atrium with the tip of the catheter

TABLE I. BLOOD OXYGEN VALUES

SUBJECT	AGE (YR.)	PER CENT OXYGEN SATURATION (CUVETTE); AIR						
		RADIAL ARTERY	I.V.C.*	S.V.C.*	R.A.*	R.V.*	P.A.*	WEDGE*
1	13	97	88‡	81	83	83	82	92
2	17	98	86	76	81	§	80	100
3	18	98	84	79	80	77	§	§
4	19	96	85	81	83	80	81	98
5	20	98	79	77	78	78	77	‖
6	21	97	84	78	81	79	79	97
7	22	98¶	65‡	74	74	79	78	‖
8	24	95	—	79	77	77	77	95
9	26	97	81	74	78	76	76	100
10**	27	97¶	78‡	69	79	69	75	99
11**	27	98	81	80	82	83	80	100
12**	27	96	87	81	78	78	77	§
13**	28	96	83	78	80	80	79	99
14**	28	98	86	77	81	77	78	100
15**	29	97¶	—	§	§	§	75	—
16**	29	99	88	75	83	80	82	98
17**	30	98	80	71	75	70	—	—
18**	30	98	76	72	76	§	79	‖
19**	31	98¶	79	77	75	75	75	—
20**	31	98	82	67	78	79	78	100
21**	32	98	82	79	80	77	76	98
22**	34	97	87	82	84	84	83	94
23**	40	98	—	77	79	80	77	100
24	41	97	—	77	81	81	77	100
25**	42	97	84	81	83	84	84	—
26	44	96	82	76	79	80	76	99
Mean		97.3	83.0	76.8	79.5	78.5	78.4	98.2
S. D.		±0.9	±3.2	±5.2	±2.8	±3.8	±2.6	±2.4
No. of samples		26	26	49	70	43	59	25
Range of individual samples		95-99	76-88	66-84	72-86	64-84	73-85	90-100

* = In this Table and in Tables II to V, I.V.C. indicates inferior vena cava; S.V.C., superior vena cava; R.A., right atrium; R.V., right ventricle; P.A., pulmonary artery; and wedge, wedge position in pulmonary artery.

†O_p = Physically dissolved oxygen (oxygen content – oxygen capacity) in arterial blood with subject breathing 95 per cent oxygen.

‡ = Samples obtained below diaphragm level, excluded from averages.

§ = Samples obtained while breathing 100 per cent oxygen only.

‖ = No flow from wedge position.

¶ = Earpiece oximeter readings.

** = Male physicians.

in a position which by fluoroscopy was apparently near the coronary sinus ostium were excluded. The mean and range of the oxygen saturation of right atrial blood excluding these samples equalled 79.5 (72 to 86) per cent, a figure intermediate between the inferior and superior caval blood saturations.

Right ventricular blood was withdrawn from both low in the right ventricle (adjacent to the tricuspid valve and from the main ventricular chamber) and high in the right ventricle (right ventricular outflow tract) and showed a mean and range of oxygen saturation of 78.5 (64 to 84) per cent, the mean value showing 1 per cent saturation less than that for right atrial blood.

The mean oxygen saturation of pulmonary arterial blood was 78 per cent. This value was similar to that of blood from the right ventricle; the range of 73 to 85 per cent was, however, smaller than the latter.

IN HEALTHY SUBJECTS

OXYGEN CAPACITY (VOL PER CENT)		TOTAL OXYGEN CONTENT VOL. PER CENT				O_2† (VOL. PER CENT) RADIAL ARTERY
		RADIAL ARTERY		PULMONARY ARTERY		
AIR	95% O_2	AIR	95% O_2	AIR	95% O_2	95% O_2
17.7	17.8	17.4	19.2	13.6	15.8	1.4
20.3	20.4	20.3	22.1	16.1	17.6	1.7
19.1	19.3	19.0	21.9	—	17.9	2.6
20.0	20.2	19.3	21.2	15.9	17.8	1.0
21.5	21.7	21.7	23.6	17.2	19.4	1.9
19.9	20.1	19.8	21.9	16.0	18.3	1.8
—	18.7	—	20.0	—	16.9	1.3
21.6	21.1	20.9	23.2	17.0	19.3	2.1
18.2	18.2	17.9	19.4	14.0	15.3	1.2
—	20.2	—	22.0	—	17.5	1.8
19.0	18.8	18.9	20.6	15.0	16.2	1.8
19.0	19.0	18.4	20.4	15.3	17.3	1.4
19.3	19.3	18.8	20.8	14.7	17.2	1.5
20.9	20.7	20.9	21.9	16.5	17.5	1.2
—	22.4	—	24.3	—	—	1.9
20.7	20.7	20.8	21.9	17.1	18.4	1.2
18.2	18.4	18.0	19.6	12.1	13.4	1.2
21.2	20.4	20.6	22.0	15.7	17.3	1.8
18.8	18.5	18.4	20.2	14.6	16.0	1.7
21.3	21.3	21.1	22.6	16.8	18.5	1.3
20.7	20.0	20.3	21.9	15.8	17.5	1.9
19.4	19.4	19.0	20.8	15.8	17.3	1.4
18.3	19.0	18.7	20.1	14.2	15.9	1.8
18.3	18.2	18.2	20.0	14.2	16.1	1.8
20.5	—	—	—	—	—	—
18.4	17.4	18.3	18.9	14.2	15.2	1.5
19.7	19.6	19.4	21.2	15.3	17.1	1.6
±1.2	±2.4	±1.0	±1.4	±1.3	±1.4	—

The lowest oxygen saturation of any sample of venous blood withdrawn from the heart or great vessels (excluding the coronary sinus and right atrium near the ostium of the coronary sinus) was 64 per cent (obtained from the right ventricle), the highest 88 per cent (obtained from the inferior vena cava). The range in oxygen saturation of individual samples in individual subjects was wide, the differences in saturations of samples withdrawn from similar sites varying from 12 per cent saturation in the inferior vena cava and the pulmonary artery to 20 per cent saturation in the right ventricle. The standard deviation of the average values from subject to subject was greatest for superior vena caval blood samples (±5.2 per cent) and least for pulmonary artery blood (±2.6 per cent).

The catheter tip was satisfactorily wedged in a peripheral branch of a pulmonary artery on 45 occasions in 22 of the 26 subjects while breathing air or 95 per cent oxygen. The criteria used in the selection of these wedge positions have been enumerated elsewhere[9]. Although an attempt was made to withdraw blood in every instance, this was successful in only 30 (66 per cent) of the instances in which the catheter was in the wedge positions. Of the 30 samples of blood obtained, a total of four (13 per cent) were less saturated with oxygen than the arterial blood. With the subject breathing room air

(Table I) 25 samples were obtained in 17 subjects, with an average oxygen saturation of 98.2 per cent. The saturations of 3 of these samples (90, 92, and 94 per cent) were below the range of arterial oxygen saturation encountered in healthy subjects. The arterial oxygen saturations recorded simultaneously by earpiece oximeter in these subjects were 95, 96, and 97 per cent, respectively.

An analysis of differences in oxygen saturation of venous blood withdrawn from the venae cavae, the right chambers of the heart, and the pulmonary artery is given in Table II, using only the differences between single pairs of blood samples withdrawn in rapid succession from different sites on the right side of the heart. By confining the analysis to consecutive samples, the subject's physiologic state was assumed to be stable during the collection of each pair of samples. Variations in the oxygen saturation of venous blood resulting from a change in cardiac output were probably minimized in this way, although variations due to sampling from streams of blood of differing oxygen saturations would presumably not be affected.

On the basis of samples withdrawn in rapid succession from the two sites, inferior vena caval blood was more highly saturated with oxygen than superior vena caval blood by a mean of 5.7 per cent (S.D. ±4.9), a statistically significant difference ($p < 0.001$). Right atrial blood was less saturated than inferior vena caval blood by a mean of 2.2 per cent (S.D. ±4.3) and more highly saturated than superior vena caval blood by a mean of 2.9 per cent (S.D. ±3.0). These differences were also statistically significant ($p < 0.01$ and 0.001, respectively). Comparison of the oxygen saturation of right atrial blood with blood from low in the right ventricle and the pulmonary artery did not show a systematic difference between these sites. Similarly there was no systematic difference between the oxygen saturations of samples from low and high in the right ventricle when compared with those from the pulmonary artery. The range of differences in oxygen saturation between the pulmonary artery and right ventricle was less than between the other sites studied.

TABLE II. AVERAGE AND RANGE OF DIFFERENCES IN PER CENT OXYGEN SATURATION OF INDIVIDUAL PAIRS OF SAMPLES OF BLOOD WITHDRAWN IN RAPID SUCCESSION FROM DIFFERENT SITES ON THE RIGHT SIDE OF THE HEART (HEALTHY SUBJECTS BREATHING AIR OR OXYGEN)

	I.V.C. –S.V.C.	R.A. –I.V.C.	R.A. –S.V.C.	R.A. –LOW R.V.	R.A. –P.A.	LOW R.V. –P.A.	HIGH R.V. –P.A.
Number of subjects	20	19	26	24	17	18	13
Number of differences	30	31	82	44	20	23	25
Mean	+5.7	–2.2	+2.9	0	–0.3	0	0
Range	-2 to +16	-11 to +6	-3 to +12	-9 to +10	-9 to +5	-3 to +4	-3 to +2
S.D.	±4.9	±4.3	±3.0	±2.9	±3.5	±1.7	±1.4
P value	< 0.001	< 0.01	<0.001	< 1.0	< 0.7	< 0.9	< 0.9

When the averages of two or more differences in oxygen saturation from paired consecutive samples of blood are compared (Table III), variations in oxygen saturation due to a changing cardiac output are minimized, and variations due to laminar flow particularly should be reduced. This reduction in variability produces a proportionate increase in the ability to detect abnormalities in the oxygen saturation of blood withdrawn from these sites. The standard deviation of the *average* differences obtained when comparing the oxygen saturation of right ventricular outflow blood with pulmonary artery blood (±1.2) was little changed from the *single* differences between these sites suggesting that mixing of returning systemic venous blood is relatively complete when it reaches the right ventricular outflow tract and pulmonary artery. Conversely the reduction in the standard deviation of the average as compared to single differences between the oxygen saturation of right atrial blood and that withdrawn from the inferior vena cava, the superior vena cava and from low in the right ventricle suggests that mixing of blood from superior and inferior venae cavae and coronary sinus was incomplete when traversing the atrium and lower part of the right ventricle.

TABLE III. AVERAGE DIFFERENCES IN PER CENT OXYGEN SATURATION BETWEEN TWO OR MORE PAIRS OF SAMPLES OF BLOOD WITHDRAWN IN RAPID SUCCESSION FROM DIFFERENT SITES ON THE RIGHT SIDE OF THE HEART (HEALTHY SUBJECTS BREATHING AIR OR OXYGEN*)

	I.V.C. –S.V.C.	R.A. –I.V.C.	R.A. –S.V.C.	R.A. – LOW R.V.	HIGH R.V. –P.A.
Number of subjects	8	9	22	14	9
Mean	+5.9	−2.2	+3.2	+0.1	+0.1
Range	+1 to +15	−7 to +2	0 to +8	−2 to +4	−2 to +2
S.D.	±4.4	±3.1	±2.3	±1.6	±1.2
P value	< 0.01	< 0.05	< 0.001	< 0.9	< 0.8

*Insufficient paired consecutive samples were available from the right atrium and pulmonary artery and the low right ventricle and pulmonary artery for comparison of average differences between these sites.

TABLE IV. DIFFERENCES BETWEEN THE AVERAGE PER CENT OXYGEN SATURATIONS AT SITES FROM WHICH MULTIPLE (RANDOM) BLOOD SAMPLES WERE WITHDRAWN WITH THE SUBJECT BREATHING AIR*

	I.V.C. –S.V.C.	R.A. –I.V.C.	R.A. –S.V.C.	P.A. –R.V.†	R.A. –P.A.	R.V.† –P.A.
Number of subjects	6	8	16	12	17	11
Mean	+5.2	−3.4	+3.0	+0.6	+1.5	+1.5
Range	+1 to +10	−9 to +1	−2 to +11	−1 to +5	−1 to +4	0 to +4
S.D.	±3.2	±2.9	±3.1	±1.8	±1.3	±1.2
P value	< 0.02	< 0.02	<0.01	< 0.3	< 0.001	<0.01

*The number of samples from each site averaged three (range 2 to 6).
†Low and high right ventricular samples both included.

In Table IV an analysis of the differences between the average oxygen saturations of samples of blood obtained in random fashion has been made. In this table the oxygen saturation at a particular site was obtained by averaging the several samples withdrawn from that site while the subject was breathing air, the number of samples from each site ranging from 2 to 6 and averaging 3. The figures were closely comparable to the average of the differences between paired consecutive samples given in Table III. Sufficient data were available in this instance to compare the average oxygen saturation of blood withdrawn from the right atrium with the combined samples from low and high in the right ventricle and also with the samples from the pulmonary artery. A small but statistically significant difference in oxygen saturation was demonstrated between the right atrium and pulmonary artery ($p < 0.001$) and the right ventricle and pulmonary artery ($p < 0.01$); pulmonary artery blood was less saturated than both right atrial and right ventricular blood by a mean of 1.5 per cent. However, when the oxygen saturations of right atrial and right ventricular blood (R.A.-R.V.) were compared in a similar manner, the mean difference of +0.6 per cent was not statistically significant ($p < 0.3$).

Variation in oxygen saturation of mixed systemic venous blood, presumably resulting in the main from a change in cardiac output in the interval between the collection of samples, was estimated in subjects with multiple samples from the pulmonary artery, assuming that blood from this site was a true sample of mixed venous blood. Thus, in 19 subjects with an average of 3 samples of blood from the pulmonary artery separated by an average interval of 37 (5 to 147) minutes, the mean of the maximal variation in oxygen saturation was 3.3 per cent and the range was 0 to 10 per cent. If it is assumed that the rate of oxygen utilization and the arterial oxygen saturation remained constant during these periods, these variations in oxygen saturation of mixed venous blood represent average maximal spontaneous variations of more than 17 per cent in cardiac output values as determined by the direct Fick method in these resting healthy subjects.

Table V. The Maximal Variation of the Differences in per cent Oxygen Saturation Between Paired Consecutive Blood Samples and Between Random Blood Samples in Subjects With 2 or More Pairs of Consecutive Samples From the Same Sites While Breathing Air*

	SAMPLES	R.A.-S.V.C.	R.A.-LOW R.V.	R.V.-P.A.
Number of subjects		13	6	7
Mean	Consecutive	2.5	2.5	2.3
	Random	7.7	7.5	7.0
Range	Consecutive	1 to 5	0 to 6	0 to 5
	Random	0 to 17	0 to 11	2 to 14
S.D.	Consecutive	±1.4	±2.5	±2.6
	Random	±5.7	±4.1	±4.5

*The time interval between random samples averaged 22 minutes (range: 2 to 115 minutes).

The value of the consecutive method of withdrawing samples from separate chambers for comparison of the oxygen saturation used almost exclusively in this study was investigated by comparing the differences in oxygen saturation between paired consecutive samples of blood from separate chambers with the differences between samples withdrawn from these sites in random fashion (Table V). As few samples were deliberately withdrawn in random fashion, samples from separate consecutive series were compared as random samples. Accordingly, in subjects with 2 or more pairs of consecutive samples from the same 2 chambers, the maximal variation for each of these differences was calculated; for example, with 3 pairs of consecutive samples from the right atrium and superior vena cava having oxygen saturations of 76 and 72, 80 and 78, 71 and 70 per cent, respectively, the differences between these paired consecutive samples equal +4, +2, +1, the differences between these samples compared in random fashion, however, equal –2, +6, +8, +10, –1, and –7, the maximal variation of the differences between consecutive samples would equal 3 per cent saturation, and between random samples 17 per cent The interval between withdrawal of the consecutive blood samples included in Table V was 30 to 60 seconds and between the random samples 22 (2 to 115) minutes One third of the random samples were separated by 2 to 8 minutes The mean of the maximal variation of differences in samples obtained consecutively varied from 2 3 to 2 5 per cent at different sites, and was much less than that for samples obtained in random fashion of from 7 0 to 7 7 per cent saturation The widest range of this maximal variation in consecutive samples was 0 to 6 per cent as compared to 0 to 17 per cent for random samples No difference in variability was found between random samples separated by 8 minutes or less and random samples separated by more than 8 minutes

COMMENT

The validity of the present analysis of oxygen saturation of blood as representative of healthy human beings rests on the absence of cardiovascular abnormalities, particularly intracardiac shunts, in the subjects included Small intracardiac shunts cannot be satisfactorily excluded by the absence of any abnormality on clinical, electrocardiographic, or roentgenologic examination of the subject Undetected veno arterial (right to left) shunts, although decreasing the oxygen saturation of the arterial and mixed venous blood, would not appreciably distort the comparative oxygen saturation of blood withdrawn from various sites in the right chambers of the heart or great vessels Should an undetected arteriovenous (left-to right) shunt be present, however, the differences in blood oxygen saturation obtained at different sites by right heart catheterization would presumably be appreciably distorted

In this study indicator dilution curves obtained after the injection of Evans blue dye (T 1824) at multiple sites within the right heart and great vessels were used to exclude hemodynamically significant vascular shunts Curves of normal contour and time components were obtained in all subjects Right to left shunts of a magnitude greater than approximately 3 to 5 per cent were considered excluded by this means[12] Left-to right shunts, recognizable by distortion of the disappearance slope of the dilution curves, are considered more difficult to detect than right to left shunts of similar magnitude[13] However, in the presence of normal cardiac output and with an accurate system of continuous recording as in the present study, left to right shunts greater than approximately 10 to 15 per cent were also considered excluded

Other cardiovascular abnormalities which might be of importance were considered excluded by measurements of intracardiac pressures and blood flow (reported elsewhere[9]) which fell within the normal range in all cases

The accuracy of the cuvette oximeter in determining the oxygen saturation of blood has been previously reported The absolute accuracy of the device varies with the oxygen saturation from ±1 to 4 per cent saturation[6, 8, 1]

The instrument has a high degree of relative accuracy, since under optimal conditions, the accuracy in the determination of differences in oxygen saturation has been reported to be ±0.1 per cent saturation.[5] Although the accuracy of the instrument decreases slightly when the lower oxygen saturations of venous blood are being recorded, this degree of accuracy is considered very *satisfactory for purposes of routine catheterization.*

The degree of accuracy obtainable in demonstrating arterialization within the right heart and associated great vessels by sampling of blood through a cardiac catheter is dependent on the constancy of the cardiac output during the collection of the samples and also on the degree of laminar flow (incomplete mixing) in the chambers from which the samples are withdrawn.

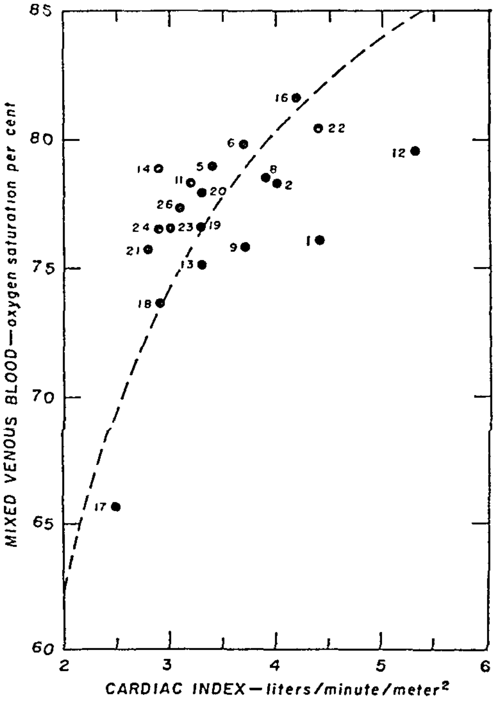

Fig. 1.—The relationship between the oxygen saturation of mixed venous blood from the pulmonary artery and the cardiac index of healthy subjects resting in the supine position. The numbers identify the individual subjects listed in Table I. The curved line was calculated on the basis of the Fick equation: Cardiac output $(\dot{Q}) = \frac{\dot{V}}{C_a - C_{\bar{v}}}$. The rate of oxygen uptake $(\dot{V})$ was assumed to be constant at the average normal level of 143 ml. per meter of body surface per minute and the arterial oxygen content (C_a) was assumed to be constant at the average normal level of 194 ml. per liter of blood (Table I).

An inverse relationship exists between the resting cardiac output and the oxygen saturation of mixed venous blood in the heart in the present subjects (Fig. 1). This relationship follows from the Fick equation $\left(\dot{Q} = \frac{\dot{V}}{C_a - C_{\bar{v}}}\right)$, for when the rate of oxygen uptake $(\dot{V})$ and arterial oxygen content (C_a) *are constant or near constant, as in the present subjects, the mixed venous* oxygen content $(C_{\bar{v}})$ will vary reciprocally with cardiac output $(\dot{Q})$.[24-26] Under the conditions of the present study the oxygen saturation of mixed venous (pulmonary artery) blood varied in oxygen content by as much as 10 per cent, presumably as the result of variations in the cardiac output and consequent changes in oxygen saturation of systemic venous blood since laminar flow (incomplete mixing) is probably insignificant when returning venous blood has reached the pulmonary artery. These spontaneous variations in oxygen saturation of venous blood make it highly desirable that samples of blood withdrawn for comparison of oxygen saturations at different sites be separated by as short an interval as practicable in the effort to minimize these effects by decreasing the time available for the changes in cardiac output which evidently occur in subjects resting in the supine position.

On the basis of the data given in Table III, utilizing the mean and twice the standard deviation of the average differences between consecutive samples the likelihood of exceeding a given range of variation in the oxygen saturation of blood from different cardiac chambers in healthy subjects can be expressed within 95 per cent confidence limits. Thus there is a 95 per cent likelihood that the differences in caval saturations (I.V.C.-S.V.C.) will not exceed a range of –3 to +15 per cent saturation points, the right atrial and inferior vena caval saturation (R.A.-I.V.C.) a range of –8 to +4 per cent saturation points, and the right atrial and superior vena caval saturation (R.A.-S.V.C.) a range of –1 to +8 per cent saturation points. Therefore, with the consecutive method of sampling, a left to right shunt (arterialization) can be considered to be present in the right atrium when the oxygen saturation of right atrial blood consistently exceeds that of inferior vena caval blood by more than 4 per cent or that of superior vena caval blood by more than 8 per cent.

For the accurate detection of arterialization within the atrium a comparison of samples of blood from both cavae with those from the right atrium is desirable. The data indicate a 95 per cent likelihood of significant arterialization in the right atrium if the oxygen saturation of a blood sample withdrawn from the mid right atrium exceeded the average saturation of samples withdrawn from the inferior and superior venae cavae by more than 4 per cent saturation. If 3 or more series of samples withdrawn in rapid succession from the inferior cava, mid right atrium, and superior cava show that the saturation of right atrial blood exceeds the average saturation of inferior and superior caval blood by more than 2 per cent, there is a 95 per cent likelihood that there is a significant source of arterialized blood in the right atrium.

A comparison of the average saturation of inferior and superior vena caval blood with that of right atrial and right ventricular blood (Tables I and II) indicates that a simple arithmetic average of superior and inferior caval blood would give a closer approximation to mixed systemic venous blood than would the ⅓ to ⅔ ratio of weighting of superior and inferior vena caval blood[27] which has been recommended for calculation of left to right shunt in cases of atrial septal defect.[22]

Arterialization within the superior or inferior vena cava, the result of an anomalous pulmonary venous connection, should be suspected when the oxygen saturation of superior vena caval blood exceeds 84 per cent or that

The instrument has a high degree of relative accuracy, since under optimal conditions, the accuracy in the determination of differences in oxygen saturation has been reported to be ±0.1 per cent saturation.[5] Although the accuracy of the instrument decreases slightly when the lower oxygen saturations of venous blood are being recorded, this degree of accuracy is considered very satisfactory for purposes of routine catheterization.

The degree of accuracy obtainable in demonstrating arterialization within the right heart and associated great vessels by sampling of blood through a cardiac catheter is dependent on the constancy of the cardiac output during the collection of the samples and also on the degree of laminar flow (incomplete mixing) in the chambers from which the samples are withdrawn.

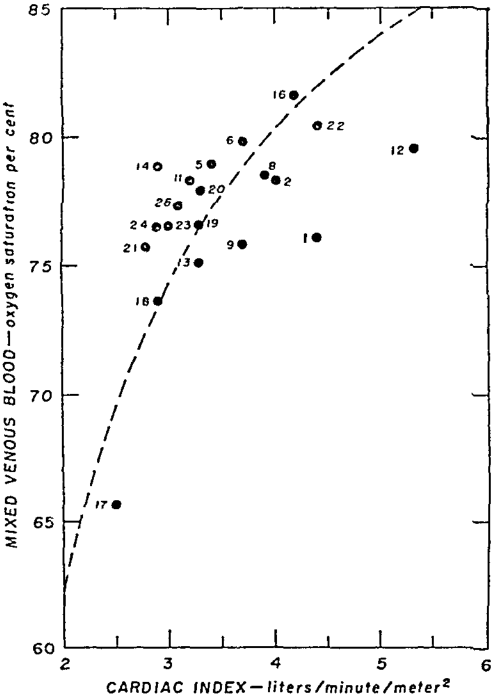

Fig. 1.—The relationship between the oxygen saturation of mixed venous blood from the pulmonary artery and the cardiac index of healthy subjects resting in the supine position. The numbers identify the individual subjects listed in Table I. The curved line was calculated on the basis of the Fick equation: Cardiac output ($\dot{Q}$) $= \frac{\dot{V}}{C_a - C_{\bar{v}}}$. The rate of oxygen uptake ($\dot{V}$) was assumed to be constant at the average normal level of 143 ml. per meter of body surface per minute and the arterial oxygen content (C_a) was assumed to be constant at the average normal level of 194 ml. per liter of blood (Table I).

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Comparison of the average oxygen saturation of blood withdrawn from all positions within the right ventricle with those obtained from the right atrium and pulmonary artery was suggestive of a slight but significant reduction in the oxygen saturation of the venous blood during its passage through the ventricle. Streamline flow of blood from the coronary sinus and thebesian veins draining into the right ventricle was considered a likely explanation of this finding although transient increases in cardiac output engendered by anxiety consequent to ectopic beats during manipulation of the catheter may be involved.

Variations in the oxygen saturation of multiple samples of mixed venous blood from the pulmonary artery from the same individual were consistent with the interpretation that the average maximal intra-individual variation in cardiac output, in these healthy subjects under conditions of supine rest, was ±17 per cent.

We wish to express our appreciation to Dr. H. J. C. Swan, Miss Lucille Cronin, and Mr. William Sutterer whose extraordinary efforts made this and the associated studies possible.[9, 10]

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DISTRIBUTION OF RADIOSULFUR, S^{35}

I. In the Human Adult

II. In the Young and the Adult Rat

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Studies using radioactive sulfur, S^{35}, as a tracer have demonstrated that sulfated compounds exist throughout the body, being especially concentrated in the connective tissue. Previous investigators have noted the distribution of S^{35} in structures of the rat, human adult, hamster, mouse, and rabbit employing the techniques of quantitative S^{35} determination, radioautography, and tissue culture.[1-8] This uptake of S^{35} into the tissues is a vital process that is probably enzymatic in nature since it does not occur in boiled cartilage nor does it occur when pure chondroitin sulfuric acid is mixed with $Na_2S^{35}O_4$.[9] By isolating chondroitin sulfate containing S^{35} from the cartilage of animals receiving $S^{35}O_4^{=}$ intraperitoneally, Dziewiatkowski in the U. S. A., and Böstrom, in Sweden, have indicated that S^{35} is incorporated into chondroitin sulfate, the essential polysaccharide of the ground substance of connective tissue.[10, 11] As animals age this organic binding of radiosulfur by the tissues has been observed to decrease gradually but markedly.[7, 10]

This study was undertaken to delineate the distribution of S^{35} in various organs, especially the connective tissue, of a human adult. Studies were also done on the quantitative distribution of S^{35} in the organs and connective tissue of young and adult rats. Some interpretation of these data is presented.

EXPERIMENTAL

Part I.—The test animals were albino rats, young animals aged about 1 month and adult animals aged 6½ to 9 months. Data were obtained from 7 adult and 3 young animals. The rats received intraperitoneally 50 μc of S^{35} as $Na_2S^{35}O_4$ in saline in a single dose. Two adult animals were given this amount of sulfur in divided doses but did not show any appreciable difference in results when compared with animals given a single dose. The rats were killed by etherization 40 hours after receiving the sulfur. Tissues were removed, cleaned, and weighed. Those that were not processed on the day of sacrifice were kept in the Deepfreeze. In general, corresponding tissues were removed from each group of animals.

Details of the procedure for ashing tissues and precipitating the sulfur as barium sulfate have been outlined in a previous report.[13] Radioactivity determinations were carried out to a probable error of 3 to 5 per cent by means of a windowless gas flow counter. Tissues that had fewer than 50 counts per minute per 100 mg. were counted to a probable error of 10 to 20 per cent.

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Since all animals were not studied at one time, different lots of S^{35} were used.* The actual content was ±20 per cent of the stated activity. No correction was made for radiodecay. Because the body size of the young animals was markedly less than that of the adult animals the total radioactivity detected in the tissues is presented with a figure representing the comparison of the sulfur-35 found in each tissue to that amount present in costal cartilage, which is easily obtained as pure cartilage.

TABLE I. S^{35} FIXATION IN TISSUES OF AN ADULT MALE RAT AND A YOUNG MALE RAT

TISSUE	AGE 5½ MONTHS		AGE 1 MONTH	
	C.P.M.*	PROPORTIONAL†	C.P.M.	PROPORTIONAL
Cartilage				
Xiphoid	880	119	8,620	125
Costal	740	100	6,910	100
Articular (tibial cap)	390	53	5,330	77
Intervertebral disc	435	63	4,700	67
Tracheal (trachea)	365	49	2,700	39
Bone				
Membranous—skull	144	19	670	10
Cancellous—tibia	130	18	780	11
Aorta	230	31	1,960	28
Tendon	115	16	560	8
Skin	145	20	780	11
Kidney	167	23	530	8
Gastrointestinal tract				
Stomach—cardiac	175	24	830	12
Stomach—pylorus	125	17	360	5
Duodenum	96	13	340	5
Ilium	119	16	460	7
Colon	114	15	623	9
Eye				
Eye coats	157	21	700	10
Lens	3	< 1	70	1
Vitreous humor	0	0		
Lung	72	10	400	6
Spleen	61	8	310	4
Liver	30	4	110	2
Pituitary	110	15	850	12
Adrenal	70	9	220	3
Pancreas	24	3	275	4
Submaxillary gland	87	12	270	4
Testis	46	6	410	6
Prostate	44	6	83	1
Seminal vesicle	23	3	480	7
Blood clot	115	16	70	1
Cerebrum	21	3	130	2
Cerebellum	30	4	180	3
Spinal cord	50	7	470	7
Muscle, cardiac	50	7		
Muscle, skeletal	15	2		
Tooth	27	4	260	4
Fat	16	2	190	3
Lymph node			360	6

*c.p.m. = counts per minute of S^{35} per 100 mg. of wet tissue.
†Proportional = relative distribution using costal cartilage fixation at 100

Part II.—The patient observed was a 51-year-old white woman who entered the hospital with widespread carcinomatosis. Several years previously she had received arsenicals and penicillin as treatment for syphilis. Thirteen days before death she was given intravenously 2.0 mc. of S^{35} as $Na_2S^{35}O_4$ in saline. Tissue samples were removed at autopsy about 9 hours after death. These samples were kept in the Deepfreeze for several days before processing and counting. No correction was made for radiodecay.

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*These were obtained from the AEC Oak Ridge National Laboratories, Oak Ridge, Tenn

RESULTS AND DISCUSSION

Structures known to contain large amounts of sulfated mucopolysaccharides were found to have the highest concentration of S^{35}. In the rat (Table I) this group includes the cartilages of the xiphoid process, the ribs, the intervertebral discs, the trachea, and the articular cartilage of the tibia. Somewhat less S^{35} was found in the membranous and cancellous bones, in the aorta, and in tendon. In structures which contain considerable amounts of connective tissue, i.e., the kidneys and gastrointestinal tract, the concentration of S^{35} was of the same magnitude as the concentration in the bone. The eyecoats, skin, and pituitary gland, as far as observed concentration of S^{35} is concerned, can also be grouped with bone. The rest of the tissues, with few exceptions, had small amounts of the isotope.

Some differences appear in data obtained from the 5 month old and 1 month old rats. The decreased fixation of S^{35} by various tissues of adult rats was due in part to the difference in size—more radiosulfur being administered proportionately to the smaller animal—and in part to the decreased fixation of S^{35} due to aging. In some structures in the adult rat the amount of radiosulfur may reflect histologic changes in the tissues, e.g., the basement membrane in the kidney becomes thicker as the animal ages. Thus the kidney reflects a higher proportional activity in sulfur uptake. Similarly, the gastrointestinal tract of the adult animal has a greater amount of radiosulfate. It is interesting to note that the seminal vesicle of the young animal has more radioactive sulfur than the seminal vesicle of the older animal, while the reverse is true of the prostate gland. These data may indicate that the biochemical changes of aging have a greater effect in some tissues than in others. The aorta in the adult and in the young animal has approximately the same sulfur ^{35} content while skin and tendon in the older animal contain appreciably more S^{35} than skin and tendon in the younger rat.

These rat data cannot be compared rigidly to values previously reported since experimental conditions differed[1, 2] especially in the time after injection of S^{35} that tissue samples were obtained. However, there is general agreement that the soft tissues are relatively low in S^{35} content. The high level of radiosulfur in the cartilage as noted here is similar to that found by Dziewiatkowski on quantitative and radioautographic study.[3]

The radiosulfur content of various tissues of the human adult (Table II) was found to contrast somewhat with the amount of S^{35} in corresponding tissues of the adult rat. Differences in the rate or period of growth of these two organisms may account for the variations. The costal and intervertebral disc cartilages of the rat have extremely high radiosulfate concentration while the costal cartilages in the human adult have relatively low S^{35} concentration. Although the tracheal cartilage in the human adult has a high level of radiosulfate, comparison with the S^{35} content of rat tracheal cartilage is difficult since the entire trachea of the rat was used for assay.

In the human adult the structures that were growing most rapidly, i.e., the tumor tissue (carcinoma of the breast) have the highest concentration of radiosulfur. This observation contrasts with that reported by Lewison and

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A MODIFICATION OF THE LATEX-FIXATION TEST FOR THE STUDY OF RHEUMATOID ARTHRITIS

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INTRODUCTION

RECENTLY, Plotz and Singer[1] have described and evaluated a so-called "latex-fixation" test as an additional serologic technique in the diagnosis of rheumatoid arthritis. An excellent correlation was found by these authors to exist between the results of this test and those observed with the sensitized sheep erythrocyte technique. The above findings, coupled with the ready availability of the test reagents (polystyrene latex particles, borate-NaCl buffer, and human gamma globulin) as well as the relative simplicity of performing the test, suggested that a further evaluation of this technique was warranted.

The purpose of the present report is to confirm and perhaps extend the observations of Plotz and Singer and to suggest a "drop" modification of the latex-fixation test which minimizes both the time required for performing the test and the quantities of reagents needed without sacrificing sensitivity.

MATERIALS AND METHODS

Latex Fixation Tests.—The essentials of the technique as described by Plotz and Singer[2] will be reviewed so that a comparison can be made between this and the proposed "drop" modification.

Latex Fixation Test (Plotz and Singer).—Two milliliters of polystyrene latex particles (Dow Chemical Co., 0.81 micron in diameter) are suspended in 20.0 ml. of water. This suspension is filtered through Whatman No. 40 paper and further adjusted with the borate buffer (pH 8.2 and containing 0.85 per cent NaCl) so that approximately 0.1 ml. of this stock latex suspension, when mixed with 10.0 ml. of buffer, will match a light transmittance of 7 per cent as measured by a spectrophotometer at 650 mμ with a red filter.

One tenth milliliter of the stock latex suspension is mixed with 9.5 ml. of buffer and 0.5 ml. of 0.5 per cent human gamma globulin (prepared by diluting lyophilized Fraction II with the borate-NaCl buffer). The resulting preparation comprises the test "antigen."

Serum samples, initially diluted 1:20 with the buffer, are further diluted by twofold increments through 1:5,120. To 1.0 ml. of each serum dilution is added 1.0 ml. of "antigen." Included in each test is an "antigen" control containing 1.0 ml. of "antigen" and 1.0 ml. of buffer.

The reactants are thoroughly mixed, by shaking the tubes, and incubated for 2 hours in a 56° C. water bath. After light centrifugation (2,300 r.p.m. for 3 minutes), the

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tubes are read for flocculation (0-4+) and the last serum dilution exhibiting evidence of aggregation is considered the titration end point. Flocculation by a serum dilution of 1:20 or greater is considered a positive test.

"Drop" Latex-Fixation Test (Rheins et al.).—The preparation of the "antigen" employed in these laboratories is similar to that just described. The notable differences are the use of polystyrene latex particles having a diameter of 1.17 micra (although of somewhat larger diameter than the particles utilized by Plotz and Singer, little difficulty has been encountered thus far because of the greater size) and the preparation of the gamma globulin. Human polio immune globulin, containing approximately 150.0 mg. of gamma globulin per milliliter, is diluted 1:30 with the borate-NaCl buffer. This dilution of gamma globulin has proved satisfactory and is analogous to the concentration of protein recommended by Plotz and Singer.

One drop of test serum is added to 1.0 ml. of buffer to effect a 1:20 dilution of the serum. From this serum stock, doubling dilutions, 1:20 through 1:5,120, are prepared by the usual serial transfer in small tubes (12 × 75 mm.), each but the first containing 2 drops of buffer. The transferring is easily accomplished with capillary pipettes. Into each tube (containing 2 drops of a serum dilution) are introduced 2 drops of the "antigen." Each titration, in addition to an "antigen" control, is accompanied by a negative serum control. This is prepared by admixture of 2 drops of a 1:20 dilution of a known negative serum and 2 drops of "antigen." After shaking, the tests are incubated for 30 minutes at either 56° C. or 37° C. (water bath).

Experience has shown that extremely mild centrifugation affords the most readable tests. Routinely, in these laboratories, this has resulted from centrifugation at 1,000 r.p.m. in an angle centrifuge. As soon as this speed is attained, centrifugation is halted and tubes are read in a near horizontal position by the oblique light from either a shaded bulb or fluorescent desk lamp. Regardless of the conditions employed for examination, the titration end point (the last serum dilution in which aggregates are evident) is determined by comparison with the negative serum control. Differences in viscosity between the greater serum concentrations of the titration and the "antigen" control sometimes introduces an element of error into the test. This is overcome to a great extent by the rigid use of the negative serum control.

By the modification just described, it is possible to perform a complete titration with 1.0 ml. of "antigen" and one drop of test serum.

A screening procedure has proved practical when large numbers of sera are to be tested. Two drops of each serum stock (1:20) and 2 drops of antigen are mixed and tested as above. The appropriate controls are included and, after incubation and centrifugation, each serum is examined and the reactivity graded from negative (–) to 4+. All positive sera and those of doubtful reactivity are titrated. Since the first serum dilution in the titration is also 1:20, an opportunity is afforded to review the doubtful sera. Although partial zones are sometimes observed (the second or third serum dilutions showing greater reactivity than the initial 1:20 dilution), no evidence of full zones has been recorded. This phenomenon is being investigated and the results will be reported at a later date.

Globulin-Coated Tanned Erythrocytes.—One-tenth milliliter of washed, packed sheep erythrocytes are tanned by the method described by Rheins and Thurston.[3] To this quantity of tanned red cells is added 1.0 ml. of human polio-immune globulin, diluted with phosphate-buffered saline (pH 7.4) so that each milliliter contains 5.0 mg. per milliliter. After incubation at 37° C. (water bath) for 30 minutes, the globulin-coated erythrocytes are thrice-washed with the saline and prepared as a 0.5 per cent suspension.

All test sera are inactivated at 56° C. for 30 minutes and adsorbed twice at room temperature with 0.4 volumes of washed, packed normal sheep erythrocytes to remove naturally occurring antibodies (each adsorption 30 minutes).

Performance of Agglutination Test.—The test is performed with quantities of reagents identical with those described for the "drop" modification of the latex-fixation test, i.e., to 2 drops of each serum dilution (1:20 through 1:5,120) are added 2 drops of the globulin treated tanned erythrocytes suspension. After mixture and incubation at 37° C. for 30 minutes, the tubes are centrifuged (1,000 r.p.m. for 2 minutes) and examined for agglutination. The usual controls are included with each test and the titration end point is determined as the last serum dilution producing visible agglutination.

EXPERIMENTAL RESULTS

Validation of the "Drop" Modification of the Latex Fixation Test.—In order to determine the reliability of a proposed "drop" modification of the latex fixation test, the following experiments were conducted: (1) Sera, shown to be positive or negative by the macromethod, were titrated by the "drop" modification. In each instance, the incubation was at 56° C. for 2 hours. (2) Titrations of positive sera were conducted at 30 minutes and at 2 hours by the "drop" modification. (3) By this technique, positive sera were titrated at both 37° C. and 56° C. for 30 minutes. Many of the same sera were employed in the three experiments.

The composite results of these tests are recorded in Table I. In no instance was a variation in titer of more than 2 dilution tubes observed. Of particular interest is the absence of false reactions among the various procedures.

TABLE I. THE EFFECT OF VARYING THE QUANTITY OF REACTANTS, THE INCUBATION TIMES, AND TEMPERATURE ON THE ACCURACY OF THE LATEX FIXATION TEST

SERA			METHOD		INCUBATION		INCUB. TEMP.		
TOTAL NO. TESTED	POSITIVE	NEGATIVE	MACRO	"DROP" MODIFICATION	30 MINUTES	2 HOURS	37° C.	56° C.	PERCENTAGE AGREEMENT
40	20	20	40	40		×		×	100
25	25			25	×	×		×	100
22	22			22	×		×		100

Based on these results, the "drop" modifications as outlined in the previous section were employed in all subsequent experiments. Incubation of the tests was conducted at either 37° C. or 56° C., whichever was most convenient.

TABLE II. RESULTS OF TESTING VARIETY OF SERA BY LATEX FIXATION TEST AND SENSITIZED, TANNED ERYTHROCYTE TECHNIQUE

TOTAL NO. OF SERA TESTED	NO. OF REACTIVE SERA	POS. BY LATEX FIX. TEST	POS. BY SENS. TAN. R.B.C.	POS. BY BOTH TESTS	POS. BY LATEX FIX. NEG. BY SENS. R.B.C.	POS. BY SENS. R.B.C. NEG. BY LATEX FIX.	PER CENT AGREEMENT
125	58	47	51	40	7	11	69

Correlation Between Results of the Latex Fixation Test and the Coated-Tanned Erythrocyte Technique.—One hundred twenty-five sera were employed in this study. Since the purpose of the experiment was to compare the results obtained with the latex-fixation test and those observed with the globulin-treated tanned erythrocytes, sera were chosen at random from those available to these laboratories. Of the total sera tested (see Table II), 47 were positive by the latex-fixation test and 51 by the sensitized, tanned erythrocyte technique. Forty sera were positive by both tests. In 7 instances, sera were positive by the latex-fixation test and negative by the sensitized, tanned red cell technique. Eleven sera reacted only with the sensitized erythrocytes. Of the 58 sera which reacted positively in one or both tests, 69 per cent (40 sera) were in agreement.

In general, the titers observed with the latex-fixation test were higher than were those seen with the sensitized red cell technique. However, the converse occurred with sufficient frequency to merit investigation as to whether these discrepancies were attributable to the techniques or reflect differences in the mechanisms of the two tests.

Serologic Reactivity of Patients Ill With Rheumatic Diseases, as Well as Other Selected Conditions.—The sera from 105 patients with a clinical diagnosis of active rheumatoid arthritis were examined by the modification of the latex-fixation test described above (Fig. 1). Ninety-two (88 per cent) gave positive reactions. In patients who had either inactive rheumatoid arthritis clinically or were thought to be in remission because of therapy, the test was positive in only 8 (66 per cent) of the sera tested. The sera from those patients with rheumatoid spondylitis in conjunction with peripheral rheumatoid arthritis were uniformly positive (100 per cent). In patients ill with other forms of rheumatic fever, diffuse collagen disease (lupus erythematosus, scleroderma) as well as spondylitis, gout, and the fibrositis syndrome, the test sera were consistently negative.

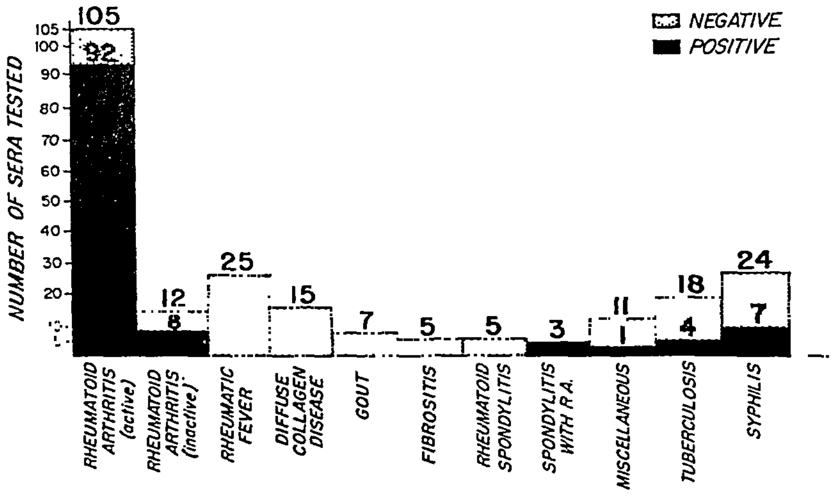

Fig. 1.—Latex-fixation reactivity of sera from patients ill with rheumatic and other selected diseases.

It should be mentioned that the latex fixation titers observed with the positive-reacting sera ranged from 1:20 through the greatest serum dilution, 1:5120. Generally the higher titers were observed in those sera from patients exhibiting more active disease.

In analyzing the rheumatic disease patients as a group the test was positive in 103 of 120 patients (86 per cent) who were considered to have rheumatoid arthritis. In a variety of other clinical conditions, nonrheumatic in nature, there was one positive serum from a case of infectious mononucleosis, 4 positive sera from 18 cases of tuberculosis, and 7 positive sera from 24 cases of syphilis. It is interesting to note that the sensitized sheep cell agglutination test also is reported to give positive reaction in these illnesses (infectious mononucleosis, tuberculosis and syphilis) in a certain percentage of cases.

DISCUSSION

In attempting to correlate the serologic results with the classification of patients with rheumatoid arthritis the following diagnostic problems were encountered. The majority of sera from patients who presented the minimal criteria required for diagnosis of probable or possible rheumatoid arthritis (as outlined by the Committee on Diagnostic Criteria of the American Rheumatism Association) gave negative reactions. Furthermore, some of these patients were under active treatment and it was difficult to evaluate the degree of activity of their disease at this time. The testing of such sera as these may have resulted in false negative reactions, thus lowering the over all percentage positivity.

Approximately 50 per cent of the sera from patients who satisfied the criteria for definite rheumatoid arthritis, but at the present time presented no clinical evidence of active disease, reacted positively. This fact suggests that either the serologic demonstration lags behind the clinical course of the disease or that in a percentage of such individuals remissions are temporary. Conversely, it is extremely difficult to determine why some of the very active rheumatoid arthritis cases gave a negative reaction.

The most difficult problem in classifying some of the patients arose in the so called diffuse collagen disease group. These represented patients who satisfied five of the criteria for definite rheumatoid arthritis, but in addition exhibited other systemic involvement which made the diagnosis of diffuse connective tissue disease the only acceptable clinical impression. The single common denominator in this group was the over all negative reactivity of their sera.

The significance of the positive reaction in the other unrelated clinical disorders is not known.

From the analysis of the data presented in this preliminary report, the following conclusions seem warranted.

The "drop" modification of the latex fixation test agrees with the results of the sheep cell agglutination test with regard to specificity. Also, the case of performing the test is an added advantage and, in addition, this test requires less technical time and fewer reagents.

In view of these observations, it is suggested that this test merits further evaluation as a serologic tool in the diagnosis, long-term management, and prognosis of rheumatoid arthritis.

The problem of reactive sera from those individuals ill with diseases other than rheumatoid arthritis suggests further investigation as to the identity of the factor or factors responsible. Furthermore, the relationship of these substances and those responsible for the positive reactivity of sera from rheumatoid arthritis patients should be established.

SUMMARY

A "drop" modification of the latex-fixation test for the serologic study of sera from patients ill with rheumatoid arthritis was described.

The results obtained with this modification apparently are in general agreement with those reported by other investigators who employed a macromethod.

It is suggested that this test merits further evaluation in the study of rheumatoid states.

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CHRONIC TOXICITY STUDIES OF HYDRALAZINE (APRESOLINE) IN DOGS WITH PARTICULAR REFERENCE TO THE PRODUCTION OF THE "HYDRALAZINE SYNDROME"

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HYDRALAZINE (Apresoline) has been used in the treatment of hypertension since 1951. In 1954, two groups of workers independently described a febrile syndrome, occurring in patients taking this drug for many months, which was characterized by fever, malaise, arthralgia, abnormal liver function tests, lupus erythematosus-like rashes, and, in the occasional case, even L.E. cells.[1, 2] These symptoms subsided on withdrawal of hydralazine or upon the administration of adrenal corticoids while maintaining the patient on hydralazine. Others have also described isolated cases of this clinical picture. Comens[3, 4] administered this drug to 8 dogs and, in 1955, reported the development of a systemic lupuslike syndrome in animals receiving 10 to 12 milligrams per kilogram for periods of 1 to 8 months. Studies by Earl[5] at Ciba Pharmaceutical Products, Inc., were not confirmatory. Since the laboratory induction of systemic lupus erythematosus (SLE) might provide a useful tool in the study of this disease, we undertook the following experiments.

MATERIALS AND METHODS

Mongrel dogs weighing from 7 to 19 kilograms were fed hydralazine in doses of from 5 to 50 mg. per kilogram per day, 7 days a week. The dosages were determined by the control period weights throughout the study and were not revised downward when weight loss occurred with the exception of Dogs 2, 4, and 10 (Tables I and II) during the latter part of the experiment. Initially the drug was given the first few animals as sugar coated tablets for a short period, but more extensively the medication was administered in powder form. First the powder was mixed with approximately 2 ounces of horsemeat but more recently it was given in capsules or forcefed if required. When the animals became too violent to be fed the material, it was injected intramuscularly. The basic ration was *Purina checkers*.

Five types of L.E. cell preparations were performed at intervals of every few weeks; these consisted of the Comens technique of utilizing serum from the dogs plus leukocytes from heparinized human blood,[3] the sieved 2 hour clot method of Zimmer and Hargraves,[6] the Snapper-Nathan ring technique,[7] and the plain heparinized method.[8] Since the description of the rotary method by Zinkham and Conley[9] and the confirmation in this laboratory of its increased sensitivity over the plain heparin method we have employed it instead of the

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TABLE I. SUMMARY OF THE COURSE

DOG	DURATION OF TREATMENT (MO.)	WT. LOSS* (KG.)	DOSE (MG./KG.)	DOSE (MG.)	TEMPERATURE†
1. Female chow (17.3 Kg.)	4.5	1.4	10-20	175-350	100.8-104.5
2. Female chow (17.0 Kg.)	12.5	3.4	10-20	170-350	102.2-102.2
3. Male wire-haired terrier (9.5 Kg.)	9.0	2.2	10-25	95-230	100.4-103.1
4. Male mongrel (10.9 Kg.)	9.0	3.2	15-20	150-200	100.7-102.2
5. Male Bedlington terrier (7.7 Kg.)	2.0	3.1	20	154	98.6-103.7
6. Female mongrel (18.6 Kg.)	1.2		25	465	101.5
7. Female mongrel¶ (13.2 Kg.)	0.5		35	465	
8. Female mongrel¶ (11.3 Kg.)	0.25		50	575	102
9. Female mongrel (11.8 Kg.)	2.0	1.4	10-15	118-160	101.8-101.5
10. Female mongrel (23.6 Kg.)	5.5	4.1	10-20	236-430	101.4-101.3

*Control and lowest level.
†Control level and maximum temperature
‡Initial sedimentation rate (Wintrobe) and maximum.

unrotated heparin technique.[10] In addition, determinations of hemoglobin, hematocrit, sedimentation rate (Wintrobe), white blood cell, and differential counts were performed routinely. The amounts of blood drawn were limited to a maximum of 20 ml. every 3 weeks to minimize blood loss. In addition, routine urinalysis and in some cases blood urea nitrogen, bilirubin, phenolsulfonphthalein and renal biopsies were performed before and after treatment.

COURSE OF HYDRALAZINE TREATED DOGS

The pattern of toxic reactions varied directly with the dosages given. Five dogs, as outlined in Tables I and II, and Fig. 1, were initially started on 10 mg. per kilogram. (Table II shows the detailed studies performed on all animals which, for the sake of abbreviation, have been summarized in Table I). Four dogs tolerated this amount without difficulty and within 4 to 8 weeks the dosage was increased to 15 mg. per kilogram. The fifth animal, Dog 3 (Table I), became cachectic and anemic on only 10 mg. per kilogram within 5 weeks (Fig. 1). His medication was omitted for 10 days and then reinstituted at a level of 5 mg. per kilogram with gradual improvement in his physical condition and laboratory findings. He was finally able to tolerate a maximum of 25 mg. per kilogram for 5 weeks by gradually increasing the dose. Dog 9 (Table I) refused the medication 5 days after the dose was increased to 15 mg. per kilogram. She was forcefed and died 2 weeks later following 24 hours of status epilepticus. The other 3 animals, Dogs 1, 2, and

OF HYDRALAZINE TREATED DOGS

HGB * (GM)	SED ‡ RATE	HT *	WBC§ (THOUS)	REMARKS‖
12 5 10 5	6 44	41 38	13 5 27 0	Preterminal convulsive seizures Dog sacrificed 5 days later
12 0 9 0	4 21	44 30	11 7 24 4	See Table II Several convulsive seizures Well at present
13 0 8 0	35 61	41 5 28	14 5 38 0	Preterminal convulsive seizures Dog sacrificed (See Fig 1)
14 5 9 0	2 53	50 30	10 1 22 2	Convulsive seizures after 4 mo of therapy and intermittently since Cachectic at present
14 0 3 8	12 83	44 10	15 5 23 0	Progressive cachexia Ten normoblasts per 100 WBC on smear Died
15 0 10 5	2 14	52 34	19 0 19 2	Violent 1 week after start of medication Sacrificed when moribund
13 0	9	45	19 9	Violent 10 days after start of medication Died
12 0	8	44	18 0	Violent 4 days after start of medication Died
12 0 11 0	6 26	44 42	13 2 14 2	Preterminal convulsions Died Last hemogram done 3 weeks before death
14 0 13 0	6 2	49 50	9 1 13 5	Convulsive seizures 3 mo after start of medication Doing well despite continued hydralazine

§Minimum and maximum levels

‖Numerous L.E. cell studies by the five techniques described in the text were performed at monthly intervals All were negative

¶Control values only Dogs were too violent to have repeat studies performed

10, tolerated 15 mg per kilogram for 5 to 7 weeks with only slight weight loss and anemia appearing in Dog 2 (see Table II) The dose was then increased to 20 mg per kilogram Within 4 days Dog 1 began to snap at the attendants and convulsed He was sacrificed Dog 2 after receiving 20 mg per kilogram for 4 weeks refused his medication and had to be forcefed Two weeks later he had convulsive seizures Dog 10 had convulsive seizures 4 weeks after increasing the dose to 20 mg per kilogram The dose was temporarily reduced in Dog 2 as shown in Table II and then cautiously increased again to 20 mg per kilogram There have been no further seizures Six weeks after increasing the medication to 20 mg per kilogram a normocytic anemia of 9 Gm per cent appeared but this corrected itself spontaneously 4 weeks later Dog 10 had no further seizures despite continuations of the medication at the same level She has not developed anemia

Dog 4 (Table I) was started on 15 mg per kilogram which he tolerated well for two months Six weeks after increasing the medication to 20 mg per kilogram he had a convulsive seizure and normocytic anemia The drug was omitted for 2 weeks and then reinstituted at a level of 15 mg per kilogram During the past 3½ months he has again received 20 mg per kilogram with partial correction of his anemia and no recent seizures

TABLE II. COURSE OF DOG 2 (FEMALE CHOW)

DATE	WT. KG.	DOSE MG./KG.	DOSE MG.	TEMP.	HGB. (GM.)	SED. RATE	HT.	WBC (THOUS.)	L.E. CELL PREP. CLOT	L.E. CELL PREP. WBC AND SERUM	L.E. CELL PREP. HEP.	L.E. CELL PREP. RING	REMARKS
2/ 6/56	17.0	10	170	102.2	12.0	4	44	12.8	0	0	0	0	Pyuria present
3/29/56	18.2	10	170	101.5	12.5	4	45	16.5	0	0	0	0	Urinalysis normal
4/ 7/56	16.8	15	260										
4/19/56		15	260										Medication given in meat
5/11/56	15.9	20	340	100.5	10.5		36	13.2					Bacilluria present
5/25/56	13.2	20	350	100.7	10.0			24.4					Dog quiet
6/ 7/56		20	350										Refused horsemeat & medication. Forcefed for 2 days.
6/16/56	13.6	20	350	100.5	10.0	21	31	20.1	0	0	0	0	Convulsive seizure. Bacilluria present
7/16/56	15.0	20	350	101.5	9.0		30	20.0					Doing well
8/ 2/56	17.7	20	350	101.5	12.0					0		0	Rotary L.E. = 0
8/13/56		20	350										Discontinued meat*
8/28/56	11.7	0	0		10.0	15	30	14.5		0			Rotary L.E. = 0. Convulsion
9/ 5/56		20	350										
9/ 9/56	10.4	0	0		9.0							0	Convulsion
9/24/56		15	260		11.0	15	36	14.0				0	Rotary L.E. = 0 Restart hydralazine
10/ 5/56	15.4	15	260	102.2									Doing well
10/16/56	15.0	13	225	102.2	11.0	8	38	15.3					Doing well
10/26/56	14.5	13	225	102.2									Eating poorly
11/ 2/56	15.0	15	225	100.5									Eating well. Urine urobilinogen pos. 1-20; neg. 1-40. Bile neg.
11/12/56	14.5	15	225	100.5									
11/26/56	13.6	15	210	100.5									
12/ 3/56	13.6	20	270	100.4	12.5		40	11.7	0	0		0	Urine normal. Bile = 0 Doing well
12/17/56	13.6	20	270	100.4									Doing well
1/17/57	13.6	20	270	100.4	9.0	7	31	16.1		0		0	Rotary L.E. = 0 Doing well
2/15/57	13.6	20	270	100.4	12.0		40	11.2					Rotary L.E. = 0 Alive and well

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*Comens suggested, in a personal communication, that since hydralazine was a chelating agent, the medication be given without meat.

It seems that tolerance to the drug was acquired by cautiously increasing the doses as shown in these 6 animals. The use of larger initial amounts discussed below produced a rapidly toxic downhill course without giving the animals time to become accustomed to the drugs.

Dog 5 (Table I) was started on 20 mg. per kilogram and within 2 months *he became cachectic, developed a normocytic anemia with 3.8 Gm. hemoglobin,* and died.

Dog 6 (Table I) was started on 25 mg. hydralazine per kilogram body weight. *Within 1 week she refused the* medication and by 2 weeks she became so violent that it was even difficult to inject the drug. The animal was sacrificed 5 weeks after starting treatment, at which time her hemoglobin was 10.5 Gm.

Dog 7 (Table I) received 35 mg per kilogram. Within one week she *refused the hydralazine and had to be forcefed and then given parenteral* material. She died 13 days after starting the drug.

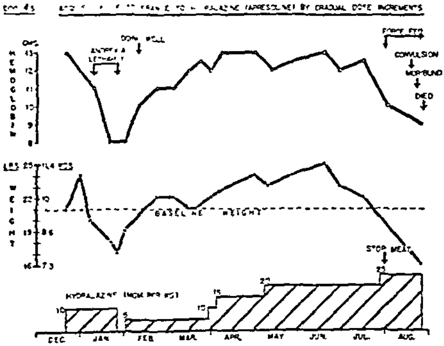

Fig. 1.—Course of Dog 3. Acquisition of tolerance to hydralazine (Apresoline) by gradual dose increments.

Dog 8 (Table I) received 50 mg. per kilogram. Two days after starting this dosage she salivated profusely and stood rigidly against the wall of her cage. *She died 6 days after starting* hydralazine in this dosage.

Most of the dogs were febrile during the latter part of hydralazine feeding. There was no evidence of arthritis at any time. (See addendum.)

LABORATORY STUDIES DURING THE COURSE OF HYDRALAZINE TREATMENT

Numerous L.E. cell preparations were performed by 5 different methods as outlined above and recorded in detail in the accompanying tables. At no time were L.E. cells seen by any of the methods. A few tart cells were noted in Dog 1. (Fig. 2). These are sometimes confused with true L.E. cells.

The anemia which appeared was normocytic. Attempts at performing a reticulocyte count failed because of inability of the dog's cells to stain in the usual manner with cresyl violet. Normoblasts were found in the blood film of Dogs 3 and 5. Quantitative serum bilirubin studies in Dog 6 before and after hydralazine administration were both normal despite the fact that his hemoglobin dropped from 15 to 10.5 Gm. in 5 weeks while receiving 25 mg. per kilogram. The indirect bilirubin rose from 0.10 to 0.25 mg. per cent but little significance can be placed on this change. The gross autopsy findings of Dogs 7 and 8 which received 35 and 50 mg. per kilogram, respectively, showed bile staining of the tissues. Unfortunately, in these animals no bilirubin determinations were made. It is probable that the mechanism of the anemia is hemolytic. Further confirmation of this was shown by the microscopic studies presented below.

Leukocytosis occurred in all the dogs. There was not one instance of leukopenia. The sedimentation rate tended to rise with progressive toxicity.

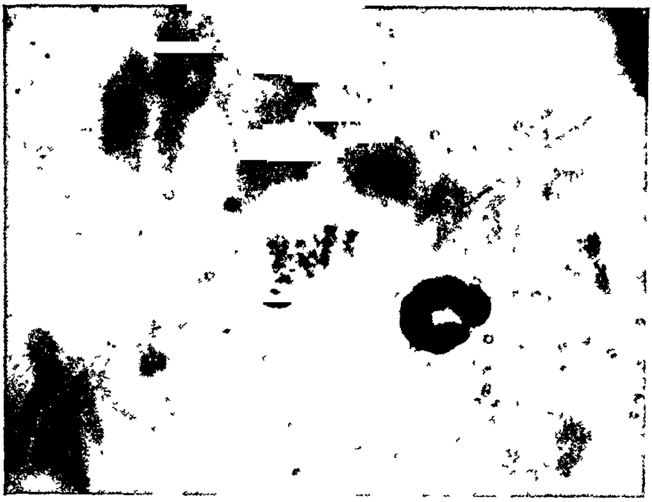

Fig. 2—Tart cell from L E preparation of Dog 1. (×1,500; reduced ¼.)

AUTOPSY FINDINGS

Four dogs died and autopsy examination was performed 5 hours post mortem on 2 and 30 hours post mortem on the other 2. Three additional animals were sacrificed by Nembutal anesthesia when obviously moribund so that fresh tissues could be obtained. The only significant gross findings were noted in the two animals who received the largest amounts of hydralazine, namely, 35 and 50 mg. per kilogram. Their viscera were bile stained. The former animal in addition had an area 2 cm. in diameter and 3 mm. deep of deeply bile stained material in the liver which appeared necrotic. The bowel wall of the other dog was hemorrhagic. The remainder of the organs including the kidneys were normal.

Microscopically the most significant changes were found in the liver and spleen. There was congestion of both organs with hemosiderosis most marked in the more severely anemic animals. No onion skin lesions of the splenic arterioles were found. Examination of the kidneys revealed increased glomerular cellularity over the control pretreatment biopsies which were available in 3 dogs. Interstitial mononuclear infiltration was noted in 6 of the 7 autopsied dogs. The infiltrate was most prominent in the subepithelial tissue of the renal pelvis and often extended along the course of the tubules. These lesions resembled low grade early human pyelonephritis. Both male and female dogs showed these changes. The females had never been catheterized, however their urine specimens were obtained by sterile transabdominal puncture of the bladder. The urine of one male showed bacilluria prior to therapy. Two other dogs had bacilluria and pyuria during treatment. Sections of kidneys from 5 other normal dogs did not show this lesion. There was no evidence of wire loop lesions. No hematoxylin bodies were seen. Brain sections revealed generalized cerebral edema in 3 out of 5 dogs and congestion of the blood vessels in 2. Numerous cortical hemorrhages were noted in Dog 9 who died in status epilepticus. The remainder of the microscopic tissue was not remarkable except for pulmonary edema in several dogs. The myocardium was normal. Bone marrow sections were available in one animal (Dog 9). Marked hyperplasia was evident in the vertebral and rib specimens.

DISCUSSION

Hydralazine in doses of 20 mg. per kilogram daily produced a progressive toxic anemia, probably hemolytic in type, terminating in death by cachexia or convulsive seizures. *These findings are similar to those of E ul*[5]. Hydralazine is chemically related to phenylhydrazine, a drug which is known to cause hemolytic *anemia*. *There has been no evidence in our studies that any* changes even suggestive of systemic lupus erythematosus can be produced. How can one then explain the "hydralazine syndrome" in human beings? It has been shown that hydralazine is a strong skin sensitizing agent[11]. The febrile hydralazine syndrome occurs in about 8 to 10 per cent of patients receiving this drug[1, 2]. The incidence of a clinical picture suggestive of systemic lupus erythematosus with the findings of L.E. cells varied from 1.4 to 3.0 per cent in two large series of cases[1]. Since the drug is a potent sensitizing agent and when injected intradermally produces delayed sensitization it is quite likely that the arthralgia and other early symptoms noted from this chemical are a serum sickness type reaction. One is led to wonder whether those patients who have such a reaction with the finding of true L.E. cells may not have latent lupus with a superimposed serum sicknesslike reaction. The propensity of lupus patients to drug sensitization is well known. Many patients with systemic lupus erythematosus present themselves with hypertension, occasionally in the malignant phase. In our series of 220 cases of SLE we have seen several whose presenting problem was malignant hypertension. Two even received hydralazine for several weeks without any ill effects.

It seems likely, therefore, that we are dealing with 2 types of "hydralazine reactions," the most common being a drug sensitivity in a patient with essential hypertension and the other type being a patient with latent SLE whose underlying disease is exacerbated by the use of hydralazine. In this latter type, the picture is typical of the underlying disease and L.E. cells are found. The disappearance of the symptoms and laboratory changes after withdrawal of a noxious agent SLE is common.

SUMMARY

Ten dogs have received hydralazine in dosages varying from 5 to 50 mg. per kilogram for as long as 12.5 months. The animals tolerated 10 mg. per day or less without difficulty and by gradually increasing the dosages tolerance can be developed to as much as 25 mg. per kilogram. The pattern and rate of toxic reactions varies directly with the dosage schedule. Utilizing 50 mg. per kilogram the animal died within a week whereas on 15 mg. per kilogram it required 3½ weeks or more. A progressive normocytic anemia, fever, anorexia, and cachexia appeared often with terminal convulsions. Leukopenia never appeared despite numerous tests. L.E. cells were not found at any time. Five different techniques were used for making the L.E. preparations. Autopsy studies showed congestion of the liver and spleen with hemosiderosis of both compatible with hemolytic anemia. Generalized cerebral edema was present. There were no renal or other changes suggestive of SLE.

ADDENDUM

Since listing the data in this paper three and one-half months have passed. Dogs 2, 4, and 10 are all alive and well on a maintenance dose of 20 mg. of hydralazine per kilogram per day. They have gained an average of one-half kilogram in weight during this period. Dog 2 has received the medication for a total of 16 months, Dog 4 for 12.5 months, and Dog 10 for 9 months. All their laboratory studies enumerated in the text, including sedimentation rate and hematocrit, are normal; L.E. cell preparations are all negative.

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CHEMICAL EVIDENCE OF TOLERANCE TO PHENOBARBITAL

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PHENOBARBITAL has been used therapeutically for many years. Its effectiveness as a sedative has long been recognized In addition, the anticonvulsive properties of phenobarbital make it a major therapeutic agent in the symptomatic treatment of grand mal and status epilepticus Thus, many patients are introduced to routine use of this drug. As a result of this prolonged therapy, habituation is not an infrequent occurrence This may result in chronic intoxication and has been described clinically by Isbell[1] Some individuals develop what seems to be a tolerance to this intoxication, and although this has been described clinically,[2-5] little chemical evidence has been offered to support it. This paper is a study of 8 cases of phenobarbital intoxication. The concurrent clinical and chemical data substantiate the thesis that tolerance does develop.

MATERIAL AND METHODS

Patients in various hospitals in Cuyahoga County (Cleveland, Ohio) who were hospitalized for acute barbiturate intoxication were studied In some of these cases, atypical chemical data suggested tolerance to the drug in these individuals In addition, several phenobarbital habituated epileptics, admitted to the hospital for the purpose of reducing or adjusting their phenobarbital therapy, were studied. Periodic blood and urine samples were taken from these patients Simultaneously, their clinical conditions were observed and objectively rated as follows

Stage I: Awake, competent, and mildly sedated

Stage II Sedated, reflexes present, prefers sleep, answers questions when roused, does not cerebrate properly

Stage III: Comatose, reflexes present

Stage IV: Comatose, no reflexes obtainable

Stage V: Comatose, no reflexes obtainable, and with circulatory and/or respiratory difficulty

These stages do not have definite limits, but blend into each other. The deep tendon reflexes were used as the criteria for the presence of reflexes Corneal, pupillary, and abdominal responses are subject to more variability in relation to the levels of anesthesia Gag and cough reflexes return at about the same time as the deep tendon reflexes, response to pain returns later The respiratory difficulty in Stage V is due to direct depression of the medullary respiratory center and is not due to the secondary pneumonia which often develops in these patients

All chemical phenobarbital determinations were done using a minor modification of the ultraviolet spectrophotometric technique described by Goldbaum[6] All chemical data

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are reported in terms of sodium phenobarbital concentration. Duplicate determinations agree within 10 per cent of each other.

Whenever possible, timed urine samples were collected to determine the renal clearance and daily excretion of phenobarbital.

RESULTS

Data obtained in the 8 patients studied are summarized in Table I. The first 4 patients were known epileptics who were hospitalized for adjustment of their epilepsy therapy. Three were withdrawn from phenobarbital and put on Hibicon and Dilantin. The fourth was continued on phenobarbital therapy.

TABLE I. PHENOBARBITAL TOLERANCE DATA

CASE	DOSE (MG./DAY)	BLOOD PHENOBARBITAL LEVELS IN VARIOUS CLINICAL STAGES				
		1	2	3	4	5
Norm		3.5	4.4	6.5	10.0	
J. M.''	1600—0	7.1	9.1			
M. H.''	128—640	5.6				
N. L.''	640—0		10.0			
R. N.''	576—0	6.7	11.5			
J. W.''		7.5	10.3			
J. U.		7.6	8.2	11.7	13.4	13.4
L. B.		7.3	8.8	10.5	12.5	17.0
N. I.''		7.7	8.2	10.6		

These cases marked " represent known epileptics. The figures under the various clinical stages (1 to 5) are the blood phenobarbital levels (mg. per 100 ml. blood) obtained as the patient was observed to enter the given clinical stage. The values opposite "Norm" represent the average blood phenobarbital concentration observed in patients hospitalized due to acute phenobarbital intoxication.

The 4 other patients were suicide attempts. Two of these were known epileptics. One other, L. B., had been on prolonged barbiturate therapy prior to this incident. In only one patient no previous history of barbiturate exposure was elicited. None of the epileptics were seriously ill at any time during their hospital stay. Two cases, L. B. and J. U., were critically ill on admission. For 5 and 7 days, respectively, constant but conservative therapy, such as that

TABLE II. BLOOD PHENOBARBITAL LEVEL WITH RESPECT TO PATIENTS' CLINICAL CONDITION

PATIENT	BLOOD BARBITURATE LEVELS (MG. PER 100 ML.) PHENOBARBITAL				
STAGE	V	IV	III	II	I
B. S.				4.0	2.4
I. B.			3.8		3.3
C. H.				4.3	
M. A.				4.6	3.6
G. W.			7.7		
R. H.			6.1		
W. Z.		11.0			
D. W.			7.4		3.9
T. L.					2.0
A. Z.					1.2
P. P.	20.6	9.2			
S. S.		9.2	5.0	4.2	3.5
E. G.			6.1		
J. D.					2.2

These data were obtained from patients (other than those reported in Table I) who were admitted to local hospitals for acute phenobarbital intoxication. The figures under the various clinical stages (1 to 5) are the blood phenobarbital levels (mg. per 100 ml.) obtained as a patient was observed to enter a given clinical stage. These data were used to determine the values used opposite "Norm" in Table I.

described by Nillson[7] and Eckenhoff,[8] was maintained by the attending physicians. At the end of these periods both these patients were in Stage II with blood barbiturate levels of 8.2 and 8.8 mg. per cent, respectively.

In all 8 patients the blood phenobarbital level was 1½ to 2 times higher than "average" for a given clinical state. The blood phenobarbital level at which these patients were able to mentate properly was approximately 7.5 mg per cent. The "average" *intoxicated patient with this blood phenobarbital* level would probably be in Stage III, comatose, with normal reflexes (Table II.)

Renal clearance of phenobarbital was studied in so far as clinical facilities allowed. The clearances were calculated in the usual way $C = \frac{U \times V}{B}$. U was the urine phenobarbital concentration and V the rate of urine flow in milliliters per minute. The blood phenobarbital concentration (B) was not corrected for protein binding. Fig. 1 shows that renal clearance is a function of the rate of urine flow.

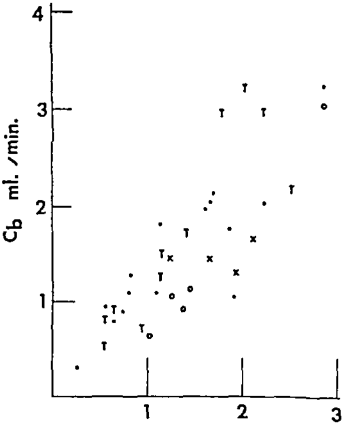

Fig. 1.—Renal clearance of phenobarbital vs. urine flow. Each symbol represents various renal clearances obtained from a given patient at various times during his hospital stay. 4 different patients were studied.

The data in Table III indicate the clearance is independent of the blood phenobarbital concentration. These data substantiate those reported by Lous.[9]

Urinary excretion of phenobarbital was also studied. The daily urinary excretion seemed to be independent of the dose. In 70 per cent of the analyses made, the daily phenobarbital excretion ranged from 80 to 160 mg. In most of the more serious phenobarbital intoxications, the average daily urinary excretion was 120 mg. The per cent of a given dose excreted daily is inve proportional to the amount ingested (Fig. 2).

In 4 patients, the daily dose of phenobarbital was known. Whenever possible, 24-hour urine samples were collected from these patients to determine the percentage of the dose excreted each day (Table IV). These data substantiate the fact that the average daily phenobarbital excretion is about 30 per cent of the ingested dose. There have been similar statements in the literature,[10, 11] but some few investigators claim this value is as high as 60 to 80 per cent.

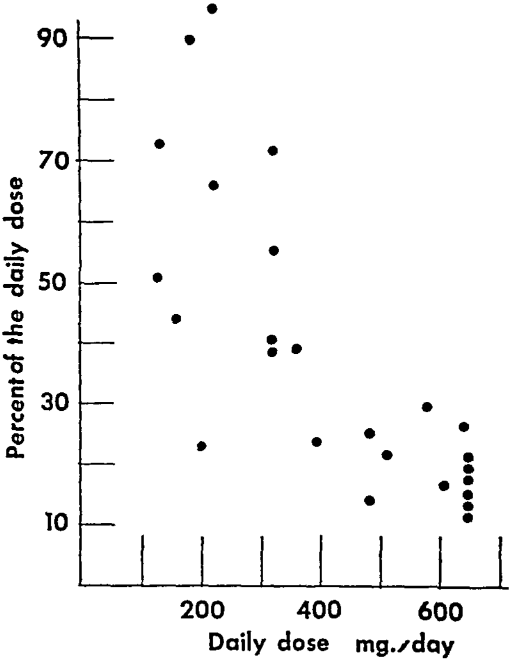

Fig. 2.—Urinary excretion of phenobarital.

TABLE III. RENAL CLEARANCE OF PHENOBARBITAL AS A FUNCTION OF BLOOD PHENOBARBITAL LEVELS

BLOOD LEVEL (MG. PER 100 ML.)	RENAL CLEARANCE (ML. PER MIN.)		
	RANGE	MEDIAN	CASES
2.0- 2.9	1.04, 1.46		2
3.0- 3.9	0.49-2.84	1.1	4
4.0- 4.9	0.73-3.1	1.3	14
5.0- 5.9	0.52; 0.80; 1.5		3
6.0- 6.9	1.2-2.2	1.7	5
7.0- 7.9	1.3-3.2	1.46	5
8.0- 8.9	0.78-3.08	1.2	8
9.0- 9.9	0.32-1.2	0.8	4
10.0-10.9	0.59, 0.83		2
11.0-11.9	0.94		1

TABLE IV. PER CENT OF DAILY PHENOBARBITAL DOSE* EXCRETED IN THE URINE

PER CENT EXCRETED	CASES
10-30	16
31-50	5
51-75	5
over 75	3

*This dose varied from 64 to 640 mg. per day.

DISCUSSION

The question of tolerance to barbiturates has been debated for many years. Much of the lack of agreement can be traced to several factors: (1) poor or loose definition of the terms tolerance and addiction; (2) differences in the time between doses, the size of doses and the species and number of animals involved in the studies. Gruber and Keyser[12] have adequately reviewed existing animal studies on this topic. Their own definitive work indicated experimental animals showed a moderate degree of tolerance to the hypnotic action of various barbiturates. There was no increased tolerance to a lethal dose (LD 50), but some cross tolerance was demonstrated. The animals lost their acquired tolerance following cessation of barbiturate administration.

Idestrom,[13] working with previously undiagnosed epileptics, found that initial daily doses of phenobarbital impaired these patients' performances of standard tests. After prolonged therapy at the same dosage, this impairment was no longer apparent.

Isbell and Fraser[2] have described human tolerance to barbiturates clinically. They indicate this tolerance is never complete. Each individual builds his own tolerance to a certain level. Any increase in the barbiturate dose beyond this, even as little as 0.1 Gm., results in marked acute intoxication.

Wright[5] states that continuous therapy with barbiturates leads to tolerance, but that such tolerance is not great. In his study he only found 2 cases with levels comparable with those in Table I, while the rest of his cases show much lower levels. He also noted tolerance to the shorter acting barbiturates.

Lous[9] studied 20 epileptic patients on daily phenobarbital therapy. Of these, 70 per cent received 200 mg. or less daily. The remainder received no more than 400 mg. daily. He noted several of these patients were in a normal state of consciousness at a serum phenobarbital level of 5 mg. per cent. Many recent investigators also indicated that most patients are somnolent at this serum phenobarbital level.

The data presented in this study (Table I) corroborate the fact that tolerance to phenobarbital does exist. The 4 epileptics studied required considerably more phenobarbital to achieve a given clinical condition than does an "average" patient. For each clinical stage described, the blood phenobarbital levels, which are a direct function of the dose, were always much higher in the 8 patients studied than those found in other patients acutely intoxicated with phenobarbital (Table II).

Acute tolerance to large doses of Pentothal was reported by Brodie.[14] Lous[15] could not demonstrate this phenomenon with either barbital or phenobarbital. For acute tolerance, those patients with high initial doses should waken with higher blood barbiturate levels than those with lower initial doses. The data on the epileptic cases in this study do not indicate acute tolerance was achieved by any of these patients. Although the actual doses were not known in 2 cases, they obviously were not the same. The actual doses in the other cases are given in Table I. They vary considerably. However, the blood phenobarbital level for Stages I and II were reasonably similar in all the cases studied (Table I).

The renal clearances of phenobarbital and its urinary excretion are the highest generally obtained for any barbiturate, with the exception of barbital. Despite this fact, the absolute values are relatively small. Thus, excretion is only a small factor in the way the body handles phenobarbital. The largest portion of the drug is metabolized, probably by the liver. Although high urine flow gives a higher clearance, it is doubtful that this type of therapy is efficacious in a seriously intoxicated person. In these cases, the urinary excretion of 80 to 160 mg. daily is not significant. Much more heroic therapy is indicated in these acute episodes.[16, 17]

SUMMARY

1. Clinical and chemical tolerance to phenobarbital is demonstrated in 8 patients who have been on phenobarbital therapy for long periods of time. These patients recovered at much higher blood levels than "average" individuals.
2. The renal clearance of phenobarbital is directly related to the urinary flow rate.
3. The renal clearance values have no relationship to the blood phenobarbital concentration.
4. An average of 30 per cent of a daily phenobarbital dose is excreted by the kidneys.
5. In 70 per cent of the analyses made, the average urinary excretion of phenobarbital varied from 8 to 160 mg. per day.

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DETECTION OF MYELOMA PROTEIN IN URINE BY A NEW QUICK METHOD

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In some patients suffering from myelomatosis the renal clearance of myeloma protein is so great in proportion to the rate of formation that the protein is not detectable in serum by electrophoresis or salting-out, owing to a very low serum concentration.

Electrophoretic demonstration of myeloma protein in urine is a relatively laborious process and consequently expensive, because prior to the electrophoresis the urinary protein has to be concentrated by salting-out and thereafter again isolated by dialysis.

Below we shall describe a simple method of detecting myeloma protein in urine, based on the differences in solubility at certain salt concentrations between myeloma protein and the protein occurring in urine from patients with nonmyelomatous renal diseases.

PRINCIPLE

In preliminary experiments we found that:

1. In urine pure myeloma protein is salted out as globulin (Fig. 1, *a*). The peak of the salting-out diagram varies from one patient to another, within a range of 30 to 60 per cent salt concentration using 3.6 mol potassium phosphate.

2. In urine nonmyeloma protein is salted out mainly as albumin. The peak of the salting-out diagram usually lies at about 75 to 80 per cent salt concentration. At 70 per cent salt concentration less than half of the total amount of protein had been precipitated in each of the cases tested (Fig. 1, *b*).

3. In cases of myelomatosis with myeloma protein in the urine the latter most often contains nonmyeloma protein as well (Fig. 1, *c*).

Whether in these cases more or less than half of *the total* protein content is salted out at 70 per cent salt concentration depends on the quantitative ratio between the myelomatous and the nonmyelomatous protein. At a low myeloma protein concentration and a high concentration of nonmyeloma protein less than half of *the total* protein may hypothetically have been salted out at a 70 per cent salt concentration.

The object of the present investigation has been to determine how often the presence of myeloma protein will cause precipitation of more than half of the *total protein* at a 70 per cent concentration of the stated salt solution, even if nonmyeloma protein is present at the same time. These preliminary results have

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been tested on 21 cases of myeloma proteinuria and the issue was compared with that of the simplest modification of the so-called Bence Jones test, *the temperature flocculation test.*

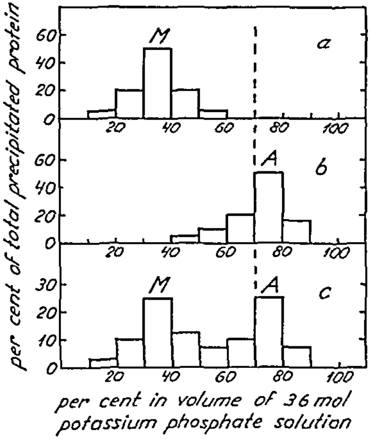

Fig 1.—Salting-out diagrams for urinary protein (outlined) *a*, Myeloma protein *b*, Nonmyeloma protein *c*, Both forms of protein in the same urine (100 per cent salt saturation = *3.6 molar equimolar monohydrophosphate and dihydrophosphate.*)

MATERIAL

1 Of 21 patients with myeloma proteinuria,* the diagnosis in 18 had been made from the finding of myeloma cells in the bone marrow.† During the development of the method, the remaining 3 were disclosed on routine examination of all the patients with proteinuria in the department. In 2 of these the diagnosis was verified by bone marrow analysis and in all 3 cases by electrophoretic analysis of the urinary protein.

Urine specimens from these 21 patients were submitted to a salting-out test as well as a temperature flocculation test In the cases where one of these two tests was negative, electrophoretic analysis of the urinary protein was carried out as well ‡

2. The urine of 50 consecutive patients was proved by Heller's test to contain protein owing to acute or chronic renal diseases of nonmyelomatous nature. Urine specimens from *these patients were submitted to a salting out test also*

3 The urine of 21 unselected patients contained protein, as revealed by Heller's test, due to acute or chronic renal diseases of a nonmyelomatous nature. Urine specimens from these patients were submitted to a temperature flocculation test.

REAGENTS

1 *Salting out Test*—

3.6 molar equimolar mono and dihydropotassium phosphate solution:

245.0 Gm. KH_2PO_4 (analytic salt)

313.6 Gm K_2HPO_4 ("rein" quality, Riedel de Haen).

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*We thank the Chiefs of the respective departments who have sent us urine from myelomatous patients

†We are greatly indebted to Dr H Gormsen of the Department of Forensic Medicine, University of Copenhagen, and Dr Charles Johansen, Prosector at the Øresundshospital, Copenhagen, for information on the myeloma patients

‡The electrophoretic analyses were carried out by Dr Niels Harboe, the electrophoretic Laboratory (Tiselius apparatus), Department of General Pathology, University of Copenhagen

The salts are dissolved in a 1000 ml. flask in distilled water up to the mark. The opalescent solution is filtered clear through hard filter paper (e.g., Schleicher & Schüll 602 eh.).

Control of the solution: A correct proportion between mono- and dihydrophosphate and a correct total salt concentration are secured by means of pH measurement and determination of specific gravity.

In the dilution of 1 ml. of salt solution to 35 ml. of distilled water the pH has been found to be 6.80 ± 0.01. The relative specific gravity against water at the same temperature has been determined at 1.376 ± 0.003.

To obtain precipitation of all the urinary protein it was necessary to work with highly concentrated salt solutions. The salt solution used is at room temperature concentrated to almost 100 per cent saturation, and the dissolution of the salts may require standing for some time and powerful shaking. Higher temperatures will increase the solubility. Since, however, it seemed in a few experiments as if the salting-out capacity increased if the solution had been heated, we chose to keep the temperature within 30° C. while dissolving the salts.

Monohydropotassium phosphate is noncrystallizable, being, therefore, unobtainable in pure form. The stated control of the solutions was accordingly necessary. Monohydrosodium phosphate, as used by S. P. L. Sørensen, is obtainable in pure crystalline form. It is, however, less readily soluble than monohydropotassium phosphate and the salt concentration at 100 per cent saturation will be so low that not all the protein in the urine can be precipitated.

The specific gravity was determined by weighing the salt solution and the distilled water in the same 20 ml. flask.

2. *Temperature Flocculation Test.*—As buffer we used the following solution:

0.07 M NaH_2PO_4
0.01 M Na_2HPO_4
pH 6.0 at ionic strength 0.1 of the solution.

PROCEDURE

1. *Salting-out Test.*—The test requires 3 ml. of clear filtered urine. The solutions are measured off into approximately uniformly calibrated test tubes, as shown in Table I, and the tubes are inverted cautiously 4 times. A positive result for myeloma protein is indicated by a more extensive precipitation in tube No. 1 than in tube No. 2. A negative result does not exclude myeloma protein (see results). If in doubt as to which of the tubes presents the most extensive precipitation, we may submit the specimens to photometry.

TABLE I. SALTING-OUT TEST

TUBE NO.	POTASSIUM PHOSPHATE SOLUTION (ML.)	H_2O (ML.)	URINE (ML.)	TOTAL VOLUME (ML.)	SALT-CONCENTRATION (PER CENT SATURATION)	CONCENTRATION OF URINE (PER CENT)
1	14	4	2	20	70	10
2	18	1	1	20	90	5

The test is inapplicable for urine that cannot be filtered clear, as well as for urine to which chemicals have been added, e.g., owing to bladder lavage.

The mixture of urine, water, and salt solution should be inverted only 4 times and should not be shaken, as shaking will cause an inconstant increase of the turbidity.

Readings can be taken immediately, and must be taken within half an hour of mixing, because standing will also cause an inconstant increase of the turbidity. For the photometry we used Pulfric's step-photometer with filter No. 6 and a light path length from 0.5 to 5.0 cm., dependent on the degree of turbidity. In the present investigations the naked-eye readings were in all cases controlled by photometric readings.

2. *Temperature Flocculation Test.*—Equal parts of the buffer and filtered clear urine, usually 2 ml. of each, are heated slowly under stirring on water bath, with a thermometer dipped in the mixture.

Flocculation at 65° C or lower temperature is reckoned as positive for myeloma protein A negative result does not exclude myeloma protein (see results)

To avoid a too rapid rise in temperature when the critical temperature of 65° C was passed we always removed the source of heat as soon as the thermometer showed 55° C and thereafter continued with very slow intermittent heating

EXPERIMENTAL

The salting out test was positive in 19 of the 21 patients with myeloma proteinuria (Table II) In the 18 cases the positive reaction was directly visible by the naked eye and confirmed by photometry In the nineteenth the reaction was doubtful when viewed directly, but photometry showed it to be positive

In the 7 cases with a positive salting out test, but a negative temperature flocculation test, the urinary protein was submitted to electrophoretic analysis which revealed myeloma protein in the urine in all cases

In one of the two patients suffering from myelomatosis with a negative salting out test the temperature flocculation test was positive Repeated salting out test on urine produced a few weeks later gave a positive result As, however, the first test was negative, possibly due to an error of the routine performance, it was set out as negative in Table II

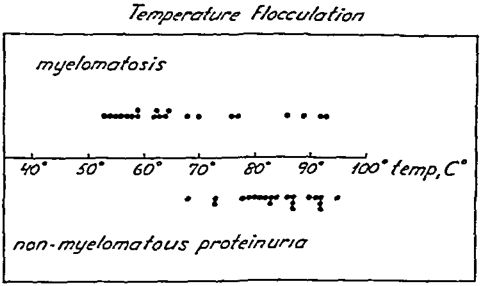

Fig 2—Flocculation temperature for urinary protein at pH 6 in patients with myelomatosis and other forms of proteinuria

In all 50 patients with renal diseases (series 2), the salting out test gave negative results when viewed directly (confirmed by photometry) While the protein present in urine from the patients of series 3 with renal diseases in no instance flocculated at temperatures under 65° C, a lower flocculation temperature was seen in urine from more than half of the patients with myelomatosis (Fig 2)

TABLE II FREQUENCY OF POSITIVE REACTIONS IN MYELOMA PROTEINURIA

NUMBER	SALTING OUT TEST	TEMP FLOCCULATION TEST "BENCE JONES"	ELECTROPHORESIS
12	Positive	Positive	Not tested
7	Positive	Negative	Positive
1	Negative	Positive	Positive
1	Negative	Negative	Positive
21	19	13	

After 3 weeks repeated testing of urine from the patient set out in the table as negative to the salting-out test and positive to the temperature flocculation test gave positive reaction to the salting-out test (see text)

If we regard a flocculation temperature under 65° C as positive with a view to myeloma protein, this reaction was positive in 13 of the 21 patients with myeloma proteinuria (Table II)

DISCUSSION

The properties of the protein described by Bence Jones[1] (1848), in his only case reported of myeloma proteinuria were, from a modern point of view, rather inaccurately explained. He only stated that by adding acid the urine became turbid, and that the turbidity disappeared by boiling and returned after cooling down. The numerous subsequent Bence Jones tests, often modified beyond recognition, work with highly different pH values and flocculation temperatures. The modifications, e.g., that of Jacobson and Wilner,[2] aimed at verifying the properties demonstrated by Bence Jones in the presence of other protein as well, such being often found, are laborious and the chance of a falsely negative result is great. They must therefore be regarded as obsolete since the introduction of the technique of electrophoresis.

The modifications based on the fact that certain myeloma proteins have a fairly low flocculation temperature are also called Bence Jones tests by some workers, though they have nothing to do with Bence Jones' original description of the properties of myeloma protein.

The temperature flocculation test, just as the salting-out test, has the great practical advantage of being easily carried out, especially compared with electrophoretic analysis of urinary protein.

The salting-out test and the temperature flocculation test both require only one reagent. For the untrained person, the salting-out test is easier to read and quicker than the temperature flocculation test, and positive findings in myeloma proteinuria are considerably more frequent, in the present series being about 90 per cent against about 60 per cent.

In our experience, a salting-out test, and possibly also a temperature flocculation test, ought to be carried out in all cases of proteinuria where a nonmyelomatous renal disease is not unquestionably present. In cases of positive results the diagnosis should be verified by electrophoretic analysis of the urinary protein.

The fact that in a medical department we disclosed within one year 3 cases of myeloma proteinuria by means of the salting-out test illustrates the value of this test.

SUMMARY

Myeloma protein can be detected in urine by a simple method based on the difference in solubility in certain salt solutions between myeloma protein and protein occurring in the urine of patients with nonmyelomatous diseases.

Among 21 patients with myeloma proteinuria the simplest modification of the "Bence Jones test," the temperature flocculation test, was found positive in about 60 per cent, whereas the salting-out test was positive in about 90 per cent. Falsely positive reactions were not observed in cases of nonmyelomatous proteinuria.

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A NEW METHOD FOR THE ISOLATION OF TUBERCLE BACILLI FROM FECAL SPECIMENS

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INTRODUCTION

THE time proved method for the isolation of tubercle bacilli employs either a sputum or gastric specimen. Before the advent of chemotherapy, this method was adequate. However, very early in the use of antituberculous drugs,[1] sputum reduction was observed to be so pronounced as to approach the disappearing point, making gastric lavage necessary. With some patients, this presents no problem. In other patients, it is a major undertaking to collect a gastric washing. *It is desirable that an alternative method be available by which cultures may be obtained.*

The use of an improved digestion method[2] and a flotation process suggested by Mudd and Mudd,[3] in 1924, have made it possible to use fecal specimens for culture of tubercle bacilli successfully. The purpose of this work was to evaluate a trisodium phosphate method for decontamination and a mineral oil method for concentration of the tubercle bacilli from fecal specimens.

MATERIALS AND METHODS

Only patients giving positive sputum cultures by standard methods were included in this study. A 2-3 Gm. fresh fecal specimen was added to a screw cap test tube containing 15 ml. of *saturated aqueous trisodium phosphate solution.* The specimen was shaken in a paint shaker for 15 minutes to obtain complete emulsion prior to incubation at 37° C. for 12 hours. Flotation of the tubercle bacilli was accomplished by the addition of 4 ml. of neutral mineral oil with an additional 5 minute shaking. The oil layer was allowed to separate out and cultures were made using only the oil layer. Three tubes of Lowenstein's medium (Jensen modification) were seeded for each specimen. Weekly readings were taken on the tubes for eight weeks. The amount of contamination was noted and the time of first appearance of tubercle bacilli on cultures recorded.

RESULTS

In all, 102 specimens were processed in the manner described above. The decontamination was very satisfactory in that only two specimens of the total were lost because of contamination. A total of 26 cultures produced growth that was typical of *Mycobacterium tuberculosis.* A representative number of these acid fast cultures were injected into guinea pigs and were found to be virulent. No particular differences in colony form or rate of growth were observed in the fecal isolations as compared to colonies from sputum or gastric specimens.

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DISCUSSION

The isolation of *Myco. tuberculosis* in 25 per cent of the fecal specimens makes this method usable under certain circumstances. Our experimental data agrees with that obtained by Petroff in 1915[4]—to isolate tubercle bacilli directly from feces is not easily accomplished. A thorough decontamination is the most important step in isolating tubercle bacilli from fecal specimens.

Since our method does not recover the tubercle bacilli in a number of the cases, it is not a substitute for the culturing of sputum or gastric specimens. However, in cases where no sputum is present and gastric lavage cannot be tolerated, fecal culture may prove of considerable use. Enzymatic destruction of the tubercle bacilli in the intestinal tract was not considered as a factor in this work. The use of a purged specimen and immediate pH adjustment to inactivate the proteolytic enzymes might be an important factor in improving this culture method.

SUMMARY

A trisodium phosphate digestion and mineral oil flotation method for recovering tubercle bacilli from fecal specimens has been described. Some methods for improving the culture recoveries are discussed.

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AN EVALUATION OF THE SAGI PLESS CANCER DETECTION TEST

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IN RECENT years considerable effort has been devoted to the development of a simple screening test for the presence of malignancy. Numerous procedures have been proposed from time to time measuring various enzymatic, immunologic, biochemical, or physical properties of blood or serum.[1] The enthusiastic initial reports have been followed almost without exception by later evaluations which have revealed such a high incidence of false positive and false negative results that the test has been discarded.[2-12]

Sagi and Pless[13] have recently described the preliminary results with a technique of differentiation of sera on phenol. These authors observed a very low incidence of false positive tests (2 of 1,500 or 0.13 per cent) and a larger but acceptable incidence of false negative tests (30 of 357 or 8.4 per cent).

The mechanism of this reaction is thought to be an interplay of the precipitating effect of diluted aqueous phenol, and the dissolving effect of concentrated phenol, upon proteins. Variations in the observed reaction are probably dependent upon qualitative and possibly quantitative differences in the serum proteins. That these differences may be specific for the presence or absence of malignant neoplasms is the basis for the use of this reaction as a screening test for cancer.

This report describes the results of an evaluation of the Sagi Pless test at a 300 bed Veterans Administration Hospital.

MATERIALS AND METHODS

Tests were performed on 101 persons, all of whom were patients in the hospital. Initial screening of the patients excluded those having certain nonmalignant diseases described by Sagi and Pless[13] as causing false positive results, i.e., uncontrolled diabetes, pernicious anemia, renal disease with nitrogen retention, viral hepatitis, and advanced cirrhosis. No patients with these diagnoses were included except 5 with mild cirrhosis (one had a weakly positive reaction and 4 had negative reactions).

In addition, Sagi and Pless noted that false positive reactions were sometimes observed in patients having recently received certain agents, such as blood transfusions, intravenous fluids, steroid hormones, anticoagulants, antibiotics, and salicylates. The widespread use of these agents made it impossible to avoid including a few such patients although an effort was made to exclude them. Radiation therapy and excisional surgery were listed as possible causes for reversion of a positive test to negative. Treatment with any of these drugs or procedures was carefully noted and the results considered separately.

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A diagnosis of malignancy was based in every case upon histologic examination of biopsy or post mortem tissues Those suspected of malignancy but in whom no pathologic confirmation was available were also listed separately

The procedure used was as originally described[13] and was followed without alteration Fasting venous blood specimens were collected, the sera separated and incubated in a water bath at 33° C for 4 hours Hemolyzed or lipemic specimens were not used. A measured amount (0.12 ml) of a phenol glycerine mixture of pH 8 0 was pipetted into each cavity of a conventional agglutination slide. Serum was then drawn up into a 1 0 ml. pipette fitted with an 18 gauge hypodermic needle with the point ground off smoothly One drop of serum was allowed to drop from the needle tip onto the phenol pool. The distance of fall was carefully maintained at 6 0 mm. by means of a clear plastic table with small holes bored therein which allowed the needle to rest on its hub while the tip was exactly 6 0 mm from the surface of the phenol The reaction of the serum drop was observed A drop which coagulated into a spherical white plug and slowly dissolved as it floated on the surface was read as negative Complete dissolution in these instances required fully 20 to 30 minutes A drop which rapidly disintegrated over the surface, dissolving immediately or within a few minutes, was read as positive

The differentiation between the positive and negative reactions for a given serum drop was unequivocal in every instance Three slide cavities were used for each specimen simultaneously In several instances, only one or two of the triplicate tests were positive while the others were negative This variable response with replicate determinations was confirmed by repeated testing of the same specimen Such results were recorded as weakly positive and were considered separately. All tests were performed and read by the same individual. The results were recorded by serial number without reference to the patient's name or clinical diagnosis

RESULTS

Of the 101 patients included in this series, 28 gave a positive test, 59 gave a negative test, and 14 were considered weakly positive Table I indicates a breakdown by type of diagnosis of those tested Of the 28 positive tests, 10 or 36 per cent were in patients without malignancy or question of malignancy and who had not received drug therapy known to cause false positive reactions If those with weakly positive reactions are included, 16 of 42 or 38 per cent were in the false positive group Table I also includes a similar analysis of those with a negative test Of these, 6 of 59 or 10 per cent were in patients with diagnosed malignancy without recent treatment which might have reverted a positive test to negative Of these 6, 3 had evident metastatic disease, the other 3 had Hodgkin's disease, bronchogenic carcinoma, and neurofibrosarcoma, respectively

When considering the series by diagnosis, 63 patients had benign conditions and 23 had malignant ones Eight were suspected of harboring malignancy which was not demonstrable and 7 were considered to have benign conditions although possibly malignant These included 5 with gastric ulcer, benign by clinical criteria but without surgical or pathologic confirmation Considering only those in whom the diagnosis was unequivocal, Table II shows that among the benign cases there were 40 with a negative test In addition, a positive test was obtained in 7 patients who had received certain drugs which might have caused a false positive result Assuming that all of the latter were falsely positive because of drugs, then 47 of the 63 or 75 8 per cent showed the correlation of a benign diagnosis with a negative test. Eleven or 15.9 per cent were unexplained false positives, or if the weakly positive results were included, the false positive percentage was 25.4.

There were 23 patients with diagnoses of malignancies with the degree of involvement ranging from minor skin malignancy in one case (basal cell carcinoma) to generalized metastases from renal, gastric, bronchial, or colonic carcinomas (Table III). With few exceptions, the patients were hospitalized as a direct result of their malignant process which was usually far-advanced. Table II also includes a breakdown of the diagnoses of malignancy. There were 6 false negatives or 26.1 per cent of the total number with malignancy. Thirteen of the 23 or 56.5 per cent showed the correlation of a positive test with the presence of malignancy. An additional 4 or 17.4 per cent were negative reactions that may be explained by preceding therapy.

Although no effort at systematic retesting was made, 9 patients had duplicate tests. Two of these were positive in one instance and negative in another. No explanation for this was apparent, both patients had benign conditions.

TABLE I. RELATION OF DIAGNOSIS TO SAGI PLESS REACTION

TYPE DIAGNOSIS	POSITIVE	WEAKLY POSITIVE	TOTAL POSITIVE	NEGATIVE
Malignancy proved	7	5	12	6
Malignancy suspected unproved	4	1	5	3
No malignancy suspected	10	6	16	40
Probably benign but malignancy a possibility	1	1	2	5
No malignancy suspected patient had received certain drugs	6	1	7	–
Malignancy proved patient had recent x-ray therapy		–	–	5
	28	14	42	59

TABLE II. SAGI PLESS REACTION IN PATIENTS HAVING UNEQUIVOCAL DIAGNOSES

TYPE REACTION	DIAGNOSIS OF BENIGN CONDITION	DIAGNOSIS OF MALIGNANT NEOPLASM
Negative	40	6
Positive	11	5
Weakly positive	5	4
Positive: result possibly related to treatment	7	4
Negative result possibly related to treatment	0	4
	63	23

DISCUSSION

It is obviously inaccurate to consider the group which received drugs as all drug-induced false positive in one instance and all genuinely positive in the next. This tends to weigh the figures in favor of the accuracy of the test. This was purposely done, however, in order to present the procedure to the best advantage, since even then it does not appear to qualify as an accurate screening or diagnostic test for cancer. Mackenzie,[14] in the discussion of the Sagi-Pless paper, mentions three series utilizing the phenol serum reaction

TABLE III. RELATION OF TYPE OF MALIGNANCY TO SAGI-PLESS REACTION

TYPE MALIGNANCY	POSITIVE	WEAKLY POSITIVE	NEGATIVE
Bronchogenic carcinoma	3*		3*
Hodgkin's disease	1	1	2
Adenocarcinoma stomach	1		1*
Lymphatic leukemia, chronic	1	1	
Basal cell carcinoma		1	
Adenocarcinoma, colon			1*
Hypernephroma			1*
Lympho-epithelioma			1
Multiple myeloma		1	
Myelogenous leukemia, chronic			1
Neurofibrosarcoma			1
Squamous cell carcinoma, mouth		1*	
Undifferentiated cell carcinoma	1*		
	7	5	11

*Metastatic disease present.

with an accuracy ranging from 92 to 96 per cent. This high level of correlation was not achieved in this series, which was able to predict the presence or absence of malignancy in only 61.6 per cent. Dunn and Greenhouse,[15] whose criteria for an acceptable screening test for malignancy are widely accepted, indicate that such a procedure should be positive in at least 90 per cent of malignant cases and should be negative in no less than 95 per cent of nonmalignant conditions.

It should be pointed out again that this series was composed entirely of hospitalized patients. Many of those with cancer had far-advanced disease. Since no outpatient examinations were included, very few cases of early malignancy were evaluated. Without long-term follow-up, it is not possible to say whether any of the presumed false positive group will turn out to have had early, undetected malignancy at the time of testing. However, each had the benefit of a complete hospital examination without evidence of malignancy being found. *In most instances, no further examinations would have been* performed without a lapse of time, even if a higher index of suspicion had resulted from the positive test. The relative absence of early malignancy may account for some of the differences noted between this study and the Sagi-Pless series, although as Homburger[9] has pointed out, it is natural to assume that a test for malignancy would show a greater degree of positive correlation in far-advanced cases.

SUMMARY

The results of an evaluation of the Sagi-Pless cancer detection test are presented. After allowing for recognizable causes of false positive and negative reactions with this test, there were 26.1 per cent false negatives and 25.4 per cent false positives. These figures greatly exceed the 10 per cent false negative and 5 per cent false positive reactions considered to be the maximum allowable for a practical screening test for cancer.

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A QUANTITATIVE MICROMETHOD FOR THE ISOLATION AND LIQUID SCINTILLATION ASSAY OF RADIOACTIVE FREE AND ESTER CHOLESTEROL

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INTRODUCTION

FOR effective utilization of radioisotopes in biologic and medical research, analytic methods are needed that permit accurate quantitative analysis and radioassay on the same sample.[1] Ideally, the procedure should not require exorbitantly large samples and should yield sufficient material for duplicate or triplicate analyses. When radioassay is performed by means of a liquid scintillation counter, the purified sample must be in a suitable state to permit solution or suspension in the scintillator solvent system. The procedure for carbon-^{14} or tritium labeled cholesterol, which is described in this report, is a modification of earlier methods.[2, 3] It was made applicable to both blood and tissue samples and was adapted for use with a liquid scintillation counter. The present technique was used to determine the specific activities of cholesterol found in 0.5 to 1.0 ml. of human plasma after the injection of 100 to 200 μc of sodium acetate 1-C$^{4-6, 14}$ as well as the cholesterol isolated from most organs of the mouse after 1.0 μc of the same precursor. In the case of human plasma, the activity of the sample containing 0.250 mg. of free cholesterol may be as low as 10^{-4} μc/mg. of carbon-^{14} or 10^{-3} μc/mg. of tritium.

MATERIALS AND METHODS

The reagents used in these experiments are all C.P. or Reagent Grade Chemical and further purification was found unnecessary. The quality of dioxane used was critical; however, Eastman Kodak Co. was found to be a reliable source of commercial dioxane. A comparison between this commercial source and the freshly distilled dioxane did not warrant the use of the tedious purification procedure. The Packard Tri-Carb liquid scintillation counter Model 314 was used to count both carbon-^{14} and tritium samples separately or simultaneously.[7]

PROCEDURE

Extraction of Plasma Cholesterol.—Fifteen volumes of acetone-alcohol (1:1) were added to 1.0 ml. of plasma at room temperature. Heating on a steam bath was unnecessary[3] but it was important to *add the solvent to the plasma* in order to precipitate the protein into a finely divided state. The mixture was filtered through Whatman No. 1 filter paper, and the filtrate collected in conical-bottom centrifuge tubes. If the solution was not clear at this point, it was refiltered. To the clear filtrate, a few drops of 10 per cent acetic acid were added.

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Extraction of Tissue Cholesterol.—Tissue samples of convenient size were rinsed in distilled water, blotted dry, and frozen in test tubes immersed in a mixture of dry ice and acetone. The weight of the frozen sample was recorded as "wet weight." The tissues (0.5 to 1.0 Gm.) were ground in 30 to 40 ml. of acetone-alcohol (1:1) solution until a fine suspension of the tissue protein was achieved. The mixture was filtered, the precipitated proteins were dried, and their weights recorded as "dry weight" (lipid free).

Isolation of the Free Cholesterol.—The cholesterol filtrates, after evaporation to more convenient volumes (10 ml.), were ready for digitonin addition. On the basis of estimates of the amount of free cholesterol in either the plasma or the tissue filtrate, approximately four times this amount of digitonin as a 1 per cent solution in 50 per cent ethanol was added. Larger amounts of reagent were avoided.[8] The mixtures were then allowed to stand overnight at room temperature so that complete precipitation would occur. After the digitonide formed, the mixture was centrifuged at 2,000 r.p.m. for 10 to 15 minutes. The supernatant fluid was then carefully decanted and the precipitate washed thoroughly by a jet stream of acetone-alcohol (1:1) solution ejected from a syringe. The washed digitonide was recentrifuged and rewashed with acetone-ether (1:2) and finally with ether alone. The washings were all combined with the first filtrate and used to estimate ester specific activity.

Isolation of Ester Cholesterol.—The above precipitation with digitonin does not remove all of the Liebermann-Burchard positive material from solution. This was demonstrated by attempting to isolate purified free cholesterol (10 mg.) by adding 4.0 ml. of 1 per cent digitonin (50 per cent alcohol) to the acetone-alcohol (1:1) solution. The filtrates, after digitonide formation and separation, were evaporated to dryness and the amount of residue was determined by the Liebermann-Burchard reaction. The results of 13 determinations showed that about 3 to 5 per cent of digitonide was in the total washing residues, or a solubility of 0.6 to 1.0 mg./100 ml. Contamination due to the solubilized digitonide and/or uncombined free cholesterol need be considered *only* when ester fractions are isolated shortly after acetate injection—the reasons being that after a sufficient time, the two specific activity curves of free and esterified cholesterol are "essentially" the same.[8, 9, 10] In those instances where the ester determinations were made, the combined washings as well as the filtrate from the "free" cholesterol were evaporated to dryness and extracted with 3 portions of hot petroleum ether (40 to 60° C.). The ether was then blown off gently with nitrogen and the ester fraction reconstituted to volume (10 to 20 ml.) with acetone-alcohol (1:1) solution. Hydrolysis of the ester was accomplished by adding 8 to 10 drops of potassium hydroxide (50 per cent) to the solution and then heating the tubes on a water bath at 40 to 50° C. for 15 minutes. The samples were then removed and allowed to cool to room temperature before acidifying to phenolphthalein with 10 per cent acetic acid. The steps after acidification are similar to those given for isolating "free" cholesterol.

Quantitative Measurement of the Digitonide.—In order to carry out the colorimetric analyses and radioactive assay on the same sample, a solvent was necessary that would dissolve the digitonide, give the Liebermann-Burchard (L.B.) color, and still retain those properties suitable for acting as a solvent in liquid scintillation counter. A system possessing these properties was composed of dioxane and phosphoric acid (ortho) in a ratio of 99:1 (v/v). Various combinations of solvent systems as well as other acids with dioxane were tested and found to be unsatisfactory.

The dioxane was therefore added to the dry precipitate, and the sample tubes were placed in a boiling water bath until solution took place. Since the standard curve has an upper limit of 0.500 mg./ml., the amount of dioxane added to each sample tube depended upon the sample size. After the digitonides were dissolved, the solutions were cooled to room temperature and adjusted to a convenient known volume. A one-milliliter aliquot was taken for quantitative determination while the other part of the sample (usually 2 ml.) was used for assaying the amount of radioactivity.

The usual ratio of sulfuric acid:acetic anhydride (1:20) with dioxane had the disadvantage of slowness in color development as well as poor sensitivity. A ratio of 1:10 developed a color with cholesterol that was sufficiently stable and gave the desired sensitivity (Fig. 1). Because of the exothermic heat produced when adding this ratio of sulfuric acid to the anhydride, the acetic anhydride was cooled in an ice bath before and during the addition of the mineral acid. The mixed L-B reagent was then allowed to come to room temperature (25 to 27° C.) and was used within one-half hour after its preparation.

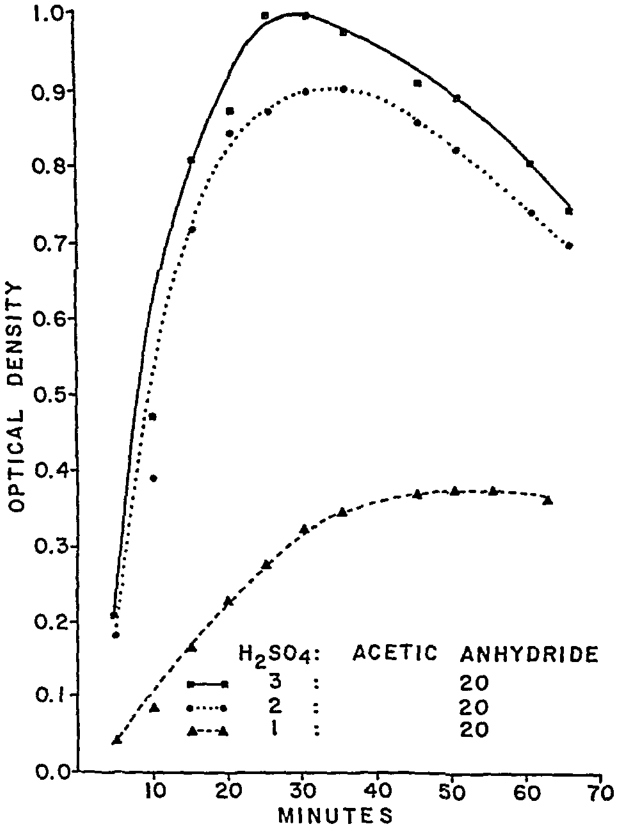

Fig. 1.—Kinetics of Liebermann-Burchard reaction: with cholesterol dissolved in dioxane, optimum operating ratio of sulfuric acid:acetic anhydride was found to be 2:20.

Standards containing various amounts of cholesterol (0.100 mg. to 0.500 mg.) dissolved in one milliliter of dioxane were prepared and mixed with 2.0 ml. of sulfuric acid-acetate anhydride mixture (1:10). As previously demonstrated,[11] *no special precautions concerning the exposure of the samples to light need be taken.* Because of the instability and slight variation in composition (acetic anhydride:sulfuric acid ratio) of the Liebermann-Burchard reagent from experiment to experiment, the maximum optical density rather than the O.D. at a particular time was used. The maximum optical density of the solutions was read at 620 mμ on a Beckman Model B Spectrophotometer. A special aluminum disk (2.4 mm. × 0.9 mm.) was machined which permitted the adaptation of the smaller volumes to this spectrophotometer. By the above procedure, a standard curve was drawn that was used for routine daily assays of cholesterol. Periodic monthly checks of the standard curve for over a year have failed to reveal any discrepancies.

TABLE I EFFECT OF PHOSPHORIC ACID CONCENTRATION ON LIQUID SCINTILLATION COUNTING

H_3PO_4 IN DIOXANE (V/V) (PER CENT)	CPM	C^{14} COUNTING EFFICIENCY (PER CENT)
0.0	567	47.1
0.5	548	45.5
1.0	503	41.8
2.0	501	41.6
4.0	463	38.5

Standard counting solution = dioxane:toluene:ETOH (5:10:1)

TABLE II EFFECT OF DIOXANE CONCENTRATION ON LIQUID SCINTILLATION COUNTING

DIOXANE (1 PER CENT H_3PO_4) (ML)	TOLUENE (ML)	ETHANOL (ML)	CARBON 14	TRITIUM
			(PER CENT EFFICIENCY)	
0	14	1.0	47.3	3.84
2	12	1.0	45.8	3.53
4	10	1.0	44.3	3.56
6	8	1.0	39.5	2.85
8	6	1.0	37.9	1.93
10	4	1.0	32.5	1.91

TABLE III DUPLICATE ANALYSES OF RADIOACTIVE CHOLESTEROL ISOLATED FROM HUMAN PLASMA

ACETATE-1-C^{14} INJECTION (HOURS)	MILLIGRAM PER SAMPLE	TOTAL COUNTS	NET CPM	CHOLESTEROL D/M/MG	μC PER GRAM
2	1.250	3,510	3,465	2,772	2.45
	1.125	3,454	3,409	3,030	2.68
4	1.000	2,736	2,691	2,691	2.38
	1.175	3,443	3,398	2,892	2.55
6	0.600	1,671	1,626	2,710	2.39
	1.450	3,540	3,495	2,410	2.13
24	1.525	2,325	2,280	1,495	1.32
	0.775	1,129	1,074	1,386	1.22

Background = 56 cpm
Carbon 14 Efficiency = 50.9 per cent

TABLE IV PRECISION AND ACCURACY OF ISOLATING AND COUNTING RADIOACTIVE CHOLESTEROL

NUCLIDE	MILLIGRAM PER SAMPLE	TOTAL COUNTS	NET CPM	CHOLESTEROL D/M/MG	RECOVERY (PER CENT)
Carbon 14*	0.436	1,391	100	524	109
	0.450	1,349	96	443	92
	0.450	1,370	98	499	104
	0.460	1,424	103	513	107
	0.460	1,419	103	513	107
				S.D. = 8.8 per cent	
Tritium†	0.440	1,713	40	1,610	105
	0.480	1,631	36	1,491	97
	0.500	1,594	34	1,332	87
	0.480	1,713	40	1,630	107
	0.490	1,674	39	1,750	114
				S.D. = 10 per cent	

Background = 39 cpm* and 46 cpm†
Samples all counted to a standard error of less than 5 per cent

MICROMETHOD FOR THE DIRECT DETERMINATION OF SERUM TRIGLYCERIDES

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INTRODUCTION

A WEALTH of information is available on the concentration of plasma cholesterol and phospholipids. However, few investigators have attempted to survey the variations of blood triglycerides in health and disease. This paucity of data is probably the result of the inadequacy of current analytical methods. For example, the solvents used for the extraction of lipids dissolve appreciable amounts of nonlipid material and extensive purification or re-extraction is required prior to the determination of total lipids by oxidative or gravimetrical methods. Furthermore, lipid-like materials such as free fatty acids, steroids, mono-, and diglycerides would be included in these nonspecific total lipid estimations. With the exception of the newer, more elaborate, chromatographic techniques, all routine determinations of triglycerides are based on the estimation of the difference between the total lipids and the sum of cholesterol, cholesterol ester, and phospholipid. As normally the triglycerides constitute only a small fraction of the total lipids, the need for a more specific method seems obvious. The present publication presents a micromethod for the direct determination of plasma, serum, or whole blood triglycerides, which is based on the quantitative measurement of the glycerol moiety of these molecules.

MATERIALS AND METHODS

Reagents.—

Fat solvents: Redistilled technical grade chloroform is satisfactory. Keep in dark bottle.

*Doucil**: Activate by heating for 4 hours at 125° C. Keep in closed bottle.

Alcoholic KOH (0.4 per cent): Dissolve 2 Gm. of reagent grade KOH in 95 per cent redistilled ethyl alcohol and dilute to 100 ml. Dilute 10 ml. stock to 50 ml. with 95 per cent alcohol on the day of use.

Sulfuric acid (0.2 N): Dilute 3 ml. of concentrated H_2SO_4 to 500 ml. with water.

Sodium metaperiodate (0.05 M): Dissolve 1.07 Gm. of $NaIO_4$ in 100 ml. of water.

Sodium arsenite (0.5 M): Dissolve 2.25 Gm. of NaOH and 5 Gm. reagent grade arsenic trioxide and dilute to 100 ml. with water.

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*Doucil is a zeolite obtained from W. A. Taylor Company, 7300 York Road, Baltimore, Md.

Chromotropic acid (0 2 per cent) Dissolve 2 Gm of chromotropic acid* (or 2 24 Gm of the sodium salt) in 200 ml of water Separately add 600 ml of concentrated H_2SO_4 to 300 ml of water, cooled in ice When cool add the diluted H_2SO_4 to the chromotropic acid solution Store in brown bottle and prepare fresh every 2 to 3 weeks

Triglyceride standard solution (0 05 mg /ml) Dissolve 0 5 Gm of commercial corn oil in chloroform and make to 100 ml in a glass stoppered volumetric flask Dilute working standard 1 to 100 with chloroform It is advisable to check the concentrated corn oil solution now and then gravimetrically to guard against increases in concentration by evaporation

PROCEDURE

Place 4 Gm of Doucil in a 28 × 130 mm glass stoppered tube, add 2 ml of chloroform, and shake Place 1 ml of plasma on top of the Doucil, mix thoroughly, and add 18 ml of chloroform Stopper the tube and shake intermittently but vigorously for about 10 minutes Filter through a coarse fat free paper and pipette 1 to 3 ml portions of the supernatant, depending on the amount of triglyceride present, into 3 glass stoppered or screw capped glass tubes (15 × 130 mm) At this point pipette three 1 ml portions of the standard corn oil solution into glass tubes and evaporate the solvent from all tubes To 2 out of each 3 standards and unknowns add 0 5 ml of alcoholic KOH (saponified sample) but to the third aliquot add 0 5 ml of alcohol (unsaponified sample) Keep all tubes at 60 to 70° C for 15 minutes Add 0 5 ml of 0 2N H_2SO_4 and place the tubes in a gently boiling water bath† for about 15 minutes to remove the alcohol After cooling the glycerol content is determined essentially according to the procedure of Lambert and Neish[1] Add 0 1 ml of periodate solution from a graduated pipette After 10 minutes, the oxidation is stopped by the addition of 0 1 ml of sodium arsenite A yellow color of iodine appears and vanishes in a few minutes Then add 5 ml chromotropic acid reagent and mix Heat for one half hour at 100° C in the absence of excessive light‡[1] After cooling, determine the optical density at 570 mμ The color remains stable for several hours

CALCULATION OF RESULTS

$$\frac{\text{O D saponified unknown}-\text{O D unsaponified unknown}}{\text{O D saponified standard}-\text{O D unsaponified standard}} \times 0\ 05 \times \frac{2\ 000}{\text{ml of chloroform extract}} = \text{mg per cent triglyceride}$$

To convert mg per cent to millimoles per liter, multiply by 0 0114

RESULTS

Standard Curve —Fig 1 shows the relationship between the optical density and the amounts of corn oil and different aliquots of a chloroform extract of plasma Thus, a good linearity is established from 0 to 200 gammas of triglyceride In several experiments, the recovery of glycerol in a known amount of oil was checked against a glycerol standard which was prepared by diluting

TABLE I GLYCEROL CONTENT OF DIFFERENT FATS (PER CENT)

	THEORETICAL	EXPERIMENTAL
Cocoa butter	10 6	10 9
Coconut oil	13 4	13 7
Corn oil	10 5	10 3
Cottonseed oil	10 8	10 5
Tripalmitin (Eastman)	11 4	10 5

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*Chromotropic acid is 4 5 dihydroxy 2 7 naphthalenedisulfonic acid

†It is advisable to keep the water level of the bath only slightly above the surface of the reaction mixture in order to avoid evaporation of the water from the tubes

‡Tubes may be heated in a boiling water or steam bath but should then be stoppered to exclude water vapors from the reaction mixture We preferred to heat the tubes in a thermostatically controlled heating block (Biochemical Associates Oakland Calif)

analytic reagent grade glycerol assayed by refractometry for water content. The glycerol content of five different fats are listed in Table I together with the theoretical amounts calculated from the average molecular weight of the fatty acids.[2] The experimental results are in good agreement with the calculated ones.

Extraction of Triglycerides From Plasma.—The extraction of lipids from plasma with the aid of Doucil and chloroform was first proposed by Forbes.[3] For the present method it became necessary to establish that complete extraction of triglycerides could be achieved without dissolving the plasma phospholipids. To investigate this problem, I^{131}-labeled triolein was emulsified with a small amount of phosphatide as described earlier[4] and added to plasma. An aliquot of plasma was then extracted by the regular Doucil-chloroform procedure and it was found that in three tests between 98 to 101 per cent of the added I^{131} triglyceride was recovered in the supernatant. Chemical analysis for phospholipid phosphorus showed that no measurable amount of phospholipid had been extracted from the plasma. A more sensitive test of this latter point was provided by the use of dialyzed rabbit plasma obtained from an animal injected with a large dose of P^{32}. Extraction of this plasma by the above procedure showed the presence of less than 0.1 per cent of plasma phospholipid P^{32} in the chloroform supernatant. The effectiveness of the chloroform-Doucil procedure was tested in yet another way. Previous investigators[5] have shown that chloroform-methanol will completely extract plasma triglycerides and that silicic acid may be used to separate the phospholipids from other lipids.[6] Thus, several plasma samples were extracted with a 2:1 mixture of chloroform-methanol. The extract was inverted with one volume of water to remove the methanol, centrifuged, and the chloroform portion poured through a silicic acid column according to Borgström.[6] I^{131} triolein emulsion added to plasma was completely

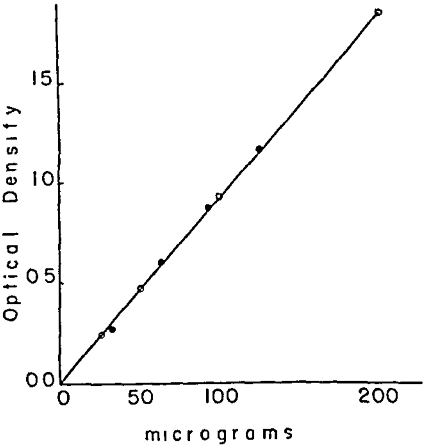

Fig. 1.—Calibration curve for corn oil (O) and Doucil-chloroform extract of dog plasma (●).

recovered by the silicic acid method. Triplicate analyses of a serum sample by the Doucil method and by the chloroform methanol silicic acid method showed good agreement between the two procedures (Table II).

Triglyceride Concentrations in Plasma of Different Species.—Plasma triglycerides determined on a group of 14 young men in the postabsorptive state are shown in Table III. The mean value of 78 mg per cent agrees well with the value of 90 mg per cent given by Peters and Man.[7] The values for dogs were obtained on mongrels bled after a 16 hour fast; the rabbits, on the other hand, had access to rabbit chow.

TABLE II. COMPARISON OF DOG PLASMA TRIGLYCERIDES DETERMINED BY DOUCIL AND BY SILICIC ACID METHODS IN MILLIGRAM PER CENT

ALIQUOT	DOUCIL	SILICIC ACID
1	33.8	34.5
2	33.7	33.5
3	33.5	35.2

TABLE III. PLASMA TRIGLYCERIDES (MILLIGRAM PER CENT)

	MEAN	RANGE	NUMBER OF CASES	STANDARD ERROR*
Normal men (postabsorptive)	78	37-124	14	7.1
Dogs (postabsorptive)	54	26-88	8	7.0
Rabbits (fed)	95	40-162	20	8.8

$$*S.E. = \sqrt{\frac{\Sigma X^2}{N(N-1)} - \frac{(\Sigma X)^2}{N^2(N-1)}}$$

DISCUSSION

The procedure outlined in the previous section consists essentially of four steps: the selective quantitative extraction of triglycerides from plasma, the complete saponification of the triglycerides, the oxidation of glycerol to formaldehyde and formic acid, and the colorimetric estimation of formaldehyde. To test the quantitative aspects of the procedure, each step had to be checked independently. Furthermore, it appeared that several alternatives were possible and the final choice of the method depends somewhat on the particular facilities available in any given laboratory. Therefore, it would seem useful to discuss briefly some additions to and possible modifications of the procedure.

The extraction of triglycerides from plasma with the aid of Doucil gives a chloroform supernatant containing not only the total amount of plasma triglyceride but the cholesterol and cholesterol ester as well. Thus, if one is interested in obtaining a quantitative estimate of plasma cholesterol in addition to the triglyceride one may use the chloroform supernatant and add to it directly acetic anhydride and sulfuric acid for the colorimetric estimation of total cholesterol.[3] If, however, one desires to determine the phospholipid in addition to cholesterol and triglyceride, an alternative procedure is suggested. One milliliter of plasma is extracted with 2:1 chloroform methanol in a 25 ml volumetric flask, the extract is filtered, and a 20 ml aliquot of the filtrate is carefully inverted with an equal volume of water and centrifuged. This gives a two phase system consisting of a solution of lipids primarily in chloroform and an aqueous-methanol supernatant. The aqueous phase is removed as quantitatively as possible by means of a syringe pipette and the chloroform layer which may still contain a little supernatant on top is poured through an activated silicic acid column.* The column is then washed with two subsequent portions of about 5 ml. of chloroform each and the eluate which contains all the triglyceride, cholesterol, and cholesterol ester is made to 25 ml. with chloroform. The phospholipid stays on the column and may be determined by estimation of the phosphorus content of a methanol eluate.[6] The procedure described here for plasma may also be applied to whole blood or to chloroform-methanol extracts of tissues. For whole blood, the Doucil procedure may also be used with good results.[8] Extraction of added I^{131} triglyceride emulsion from whole blood with chloroform in the presence of Doucil was quantitative and a comparison between the triglyceride content of whole blood as determined by the Doucil procedure and the silicic acid column gave agreement which was as good as that described for plasma.

The proposed saponification procedure was arrived at by the following experiments: saponification of small amounts of triglycerides with aqueous alkali of various concentrations led to unsatisfactory recovery of glycerol; saponification with alcoholic KOH with subsequent evaporation of alcohol led to considerable loss of glycerol. Indeed, when alcohol was distilled from an alcoholic glycerol solution in a boiling water bath, considerable amounts of glycerol appeared in the distillate. Such loss of glycerol was not observed when the alcoholic glycerol solution was diluted with an equal volume of water. Failure to remove the ethanol before oxidation with periodate produced a turbidity during the heating with the chromotropic acid. The development of this turbidity seems to be related to the presence of cholesterol since it did not occur in standard corn oil solutions to which no cholesterol had been added. To avoid these difficulties, saponification was carried out in 95 per cent ethanol below the boiling point of alcohol. A 15-minute heating between 60° and 70° C. appeared to be satisfactory since no difference in glycerol recovery was observed when corn oil was saponified at 65° C. from 5 to 60 minutes.

The determination of the presaponification blank (which includes the reagent blank) increases the specificity of the method and prevents errors from the possible presence of formaldehydogenic steroids or monoglycerides in the lipid extract. Normally the presaponification blank does not exhibit the typical formaldehyde chromotropic-acid color. Thus, one may easily distinguish between this nonspecific color of the blank and the presence of formaldehydogenic materials in the lipid extract. At this point, it should be noted that the presence of α-monoglyceride in the lipid extract would produce a presaponification color which is half that produced after saponification, whereas the presence of β-monoglycerides and diglycerides would not be detected in this manner. So far, we have not found any instances where the color in the unsaponified samples of blood or plasma resembled that produced by glycerol.

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*A convenient way of preparing silicic acid columns for serial determinations is the use of 10-ml. syringe barrels in which a small plug of glass wool is inserted, followed by a 1-Gm. portion of 1:1 mixture of activated silicic acid (Mallinckrodt) and Supercel (Johns-Manville)

SUMMARY

A microprocedure for the direct determination of triglyceride concentrations in biologic specimens is presented. The method depends on the quantitative removal of phosphatides from the sample and the subsequent determination of esterified glycerol. The procedure has been tested on whole blood and plasma.

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A TECHNIQUE FOR THE SAFE HANDLING OF COCCIDIOIDES IMMITIS CULTURES

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INTRODUCTION

FOR at least ten years there have been repeated warnings of the danger to laboratory personnel in the handling of cultures of *Coccidioides immitis.*[1-7] Smith and Harrell[2] reported 6 cases involving laboratory workers as a consequence of accidental contamination of the outside of flasks with liquid cultures of this fungus. Looney and Stein[3] reviewed 33 cases of accidental infections described in the literature and reported one new case of disseminated infection in a laboratory worker. Sulkin and Pike[4] analyzed a total of 1,342 laboratory infections, of which 63 were caused by fungi, principally *C. immitis.*

Despite the hazards involved, there are occasions when many laboratories must handle cultures of *C. immitis.* These instances are no longer restricted to the institutions located in the recognized endemic areas but may occur in any geographical location, since individuals who have contracted the infection may not exhibit disease until after they have left the endemic region.[3, 8-13] Evans and his associates[13] warned that coccidioidomycosis may be encountered by physicians in any part of the United States. While all microbiological laboratories must be aware of the dangers inherent in the handling of cultures of *C. immitis*, this is particularly true for those clinical laboratories where this fungus has been encountered only infrequently or not at all. It is in these laboratories where inexperience with *C. immitis* is most likely to be associated with lack of adequate facilities for handling such cultures safely.

It is the purpose of this report to describe a technique which will enable any laboratory to isolate and identify *C. immitis* with safety.

MATERIALS AND METHODS

All strains of *C. immitis* used were obtained from the stock culture collection of the Mycology Research Laboratory, San Fernando Veterans Administration Hospital. These strains were selected as representative of a range of characteristics, such as, recently isolated cultures and those which had been subcultured for varying periods, the presence or absence of arthrospores, and the presence or absence of aerial hyphae. For preliminary testing a strain of Penicillium was used.

Four media were used: Sabouraud's dextrose agar; Sabouraud's dextrose agar with added antibiotics; Blood Agar Base (Difco); Blood Agar Base with added antibiotics. The antibiotics were sterilized separately by filtration and incorporated into the sterile

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media after they had been melted and cooled to 50° C. The concentrations used were: penicillin, 20 units per milliliter; streptomycin, 40 units per milliliter, cycloheximide, 0 5 mg per milliliter [11]

After adding the antibiotics, the media were poured into sterile 2 ounce prescription bottles, each fitted with a screw cap containing a rubber diaphragm (Fig 1). The bottles were slanted and the medium allowed to harden. When ready for inoculation, the bottles and the stock cultures of *C immitis* were placed in a transfer chamber which was then sealed. The front of this chamber contained two rubber gloves with an attached accordion-type sleeve to permit relatively free arm movement within the chamber. All culture transfers were made in this manner. Air was circulated through the chamber by means of an exhaust fan Both incoming and outgoing air were filtered, and a constant cultural check was maintained on the exhaust air to determine whether or not spores passed the filter.

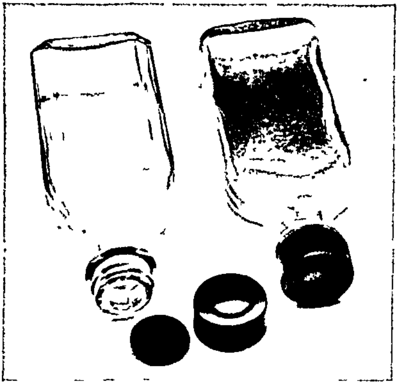

Fig 1—Two culture bottles with rubber-diaphragm screw caps are shown One is empty and disassembled, the other contains agar and is assembled ready for use

Fluid was added to or withdrawn from the culture bottles with Luer Lok needles and syringes. The rubber diaphragm was painted with 3 per cent tincture of iodine before insertion of the needle. Sterile cotton, soaked in 70 per cent aqueous ethanol, was wrapped around the needle and the point of penetration in the rubber diaphragm After fluid was drawn into the syringe, the needle and syringe were extracted very slowly, and, after the needle point had cleared the diaphragm, it was held tightly within the alcohol soaked cotton to prevent any aerosol dispersal of infectious material into the surrounding air.

Petri plates containing Sabouraud's dextrose agar were exposed continuously throughout all experiments These culture dishes were placed both in the transfer chamber and in the laboratory

All techniques were done first with the strain of Penicillium and then repeated with the *C immitis* cultures.

RESULTS

Testing for Disposal of Fungous Spores During Transfer Operations—Using the Penicillium strain 5 transfers were made from a 2 week-old stock culture to fresh media. During the operation 6 plates of media were exposed inside the chamber, and the exhaust air from the chamber was allowed to impinge on an additional plate. After completion of the transfers, the exposed plates were incubated at 30° C. for 2 weeks. The experiment was repeated 10 times.

TABLE I. THE DISPERSION OF PENICILLIUM SPORES DURING TRANSFERS FROM STOCK CULTURE TO FRESH MEDIA USING A THIN TRANSFER NEEDLE

EXPERIMENT	COLONIES OF PENICILLIUM RECOVERED	
	INSIDE CHAMBER	FROM CHAMBER EXHAUST
1	6	0
2	3	0
3	0	0
4	5	0
5	2	0
6	0	0
7	1	0
8	1	0
9	4	0
10	7	0
	29	0

As recorded in Table I, a total of 29 colonies of Penicillium were recovered from plates exposed inside the chamber but none was found on plates exposed to the chamber exhaust, indicating efficient removal of spores from the air stream by the filter. It had been noted during the transfer operations that occasionally the thin wire needle seemed to "whip" slightly as it was withdrawn after cutting out a piece of agar containing growth. It was suspected that this could result in dispersing some spores into the air. The experiment was repeated, therefore, using a stiff dissecting needle, and the results are recorded in Table II. While the number of colonies of Penicillium was reduced considerably, it should be noted that, even with the most careful technique, some spores still escape into the environment.

TABLE II. THE DISPERSION OF PENICILLIUM SPORES DURING TRANSFERS FROM STOCK CULTURE TO FRESH MEDIA USING A STIFF DISSECTING NEEDLE

EXPERIMENT	COLONIES OF PENICILLIUM RECOVERED	
	INSIDE CHAMBER	FROM CHAMBER EXHAUST
1	0	0
2	0	0
3	1	0
4	0	0
5	2	0
6	0	0
7	0	0
8	0	0
9	1	0
10	1	0
	5	0

Sterilization of the Chamber.—Two sets of 10 cultures each were inoculated with Penicillium in the transfer chamber. On one set the caps were screwed down tightly while on the other set the caps were loose. Approximately 10 ml of 37 per cent formaldehyde were poured into a beaker. After 24 hour exposure to formaldehyde vapor, all cultures were removed and incubated at 30° C. for 2 weeks. The experiment was repeated 10 times.

In the bottles which had been capped tightly, the Penicillium grew abundantly, while there was no growth in the bottles with loose caps. The same results were obtained when the experiment was repeated with strains of *C. immitis*.

In view of the results obtained from these two sets of experiments, it was felt that further work with cultures of *C. immitis* could be accomplished without endangering laboratory personnel.

Handling of Cultures of C. immitis.—Ten cultures of *C. immitis* were made on each of the four media. The bottles were capped tightly and exposed to formaldehyde for 24 hours. All 40 cultures were incubated at 30° C. for 2 weeks. Excellent growth was obtained in each culture.

Varying amounts of three concentrations of formaldehyde were injected through the rubber diaphragm of the tightly closed caps of 9 cultures of each of the 4 sets of media. One bottle of each medium was kept as a control. After 24 and 48 hours at 37° C., transfers were made from each culture to fresh media and incubated at 30° C. for one month. The results are recorded in Table III. All nonexposed control cultures were positive. All subculture bottles which were negative were reinoculated at the end of the experiment with a viable culture and growth occurred in each case.

TABLE III. GROWTH OF SUBCULTURES OF C. IMMITIS (C 25) AFTER EXPOSURE TO VARYING AMOUNTS OF FORMALDEHYDE FOR 24 AND 48 HOURS

	PER CENT CONCENTRATION OF FORMALDEHYDE	GROWTH ON SUBCULTURE					
		AFTER 24 HOUR EXPOSURE			AFTER 48 HOUR EXPOSURE		
		VOL. FORMALDEHYDE ADDED			VOL. FORMALDEHYDE ADDED		
GROWTH ON		0.5 ML	2.0 ML	8.0 ML	0.5 ML	2.0 ML	8.0 ML
Sabouraud's							
Dextrose	0.5	+	–	–	+	–	–
Agar	2.0	–	–	–	–	–	–
(SDA)	8.0	–	–	–	–	–	–
SDA							
with	0.5	+	+	–	+	–	–
Anti-	2.0	–	–	–	–	–	–
biotics	8.0	–	–	–	–	–	–
Blood							
Agar	0.5	+	–	–	+	+	–
Base	2.0	–	–	–	–	–	–
(BAB)	8.0	–	–	–	–	–	–
BAB							
with	0.5	+	+	–	+	–	–
Anti-	2.0	–	–	–	–	–	–
biotics	8.0	–	–	–	–	–	

This experiment was repeated using 10 strains of *C. immitis*, 0.5 ml. amounts of the 3 concentrations of formaldehyde, and Sabouraud's dextrose agar. All cultures were done in triplicate. Representative results are recorded in Table IV. All cultures exposed to 8 per cent formaldehyde were sterilized, while the results were variable with the 0.5 and 2 per cent concentrations.

TABLE IV. GROWTH OF SUBCULTURES OF TEN STRAINS OF C. IMMITIS AFTER EXPOSURE TO 0.5 ML. OF VARYING CONCENTRATIONS OF FORMALDEHYDE FOR 24 AND 48 HOURS

PER CENT CONCENTRATION OF FORMALDEHYDE	GROWTH ON SUBCULTURE	
	AFTER 24 HOUR EXPOSURE	AFTER 48 HOUR EXPOSURE
0.5	26/30*	9/30
2.0	2/30	0/30
8.0	0/30	0/30

*Numerator represents the total number of subcultures which were positive for *C. immitis* and the denominator the total number of subcultures made.

A single culture of each of the 10 strains was made on Sabouraud's dextrose agar and on Blood Agar Base. After incubation at 30° C. for 2 weeks, slides were made from each culture in 20 per cent NaOH in glycerin-water (50:50). The cultures were killed by exposure to 0.5 ml. of 8 per cent formaldehyde for 24 hours and a second set of slides was made. The microscopic appearance of a culture which had been killed by formaldehyde was comparable to that of the corresponding viable culture.

Suspensions of viable material were made from cultures grown in the bottles with rubber-diaphragm screw caps. Five milliliters of saline were injected through the diaphragm after the cap was closed tightly. The bottle was shaken vigorously and the resulting suspension was withdrawn with a Luer-Lok needle and syringe. Extreme care was taken to prevent the escape of an aerosol, particularly at the moment the needle was withdrawn from the diaphragm. No colonies of *C. immitis* were detected on plates exposed in the area during the operation. No difficulty was encountered in obtaining suspensions from those cultures which produced aerial hyphae. Frequently, however, macroscopically visible particles would separate from the agar and from the colony itself. These could be avoided by inverting the culture, allowing the particles to settle, and inserting the syringe needle to a level above the sediment before withdrawing any fluid. Suspensions with one million or more cells per milliliter could be prepared without ever opening the culture. Such suspensions were satisfactory for injecting animals to produce coccidioidomycosis as the final step in the identification of *C. immitis*.

DISCUSSION

Since it is quite possible that any clinical laboratory might isolate a culture of *C. immitis* at some time, it is imperative that each laboratory have the facilities available to handle such cultures with safety for the personnel involved. Not many laboratories are equipped with completely sealed transfer chambers for this purpose, and it would be impractical for them to maintain such an apparatus on the chance that it would be used only rarely. This disadvantage can be overcome, however, by using an inexpensive bottle fitted with a rubber-diaphragm screw cap for culturing specimens which might contain *C. immitis*. As a general rule, these bottles could be used for culturing any specimen which might contain microorganisms capable of producing pulmonary disease in man. The greatest danger, however, lies in the possibility of accidentally breaking the container of such cultures. The 2-ounce prescription bottles used here are quite sturdy and have remained undamaged after dropping to a concrete floor. Nevertheless, an unbreakable plastic container, which could be sterilized, would be even better.

The following procedure is suggested specifically for specimens in which *C. immitis* may be encountered. Portions of the specimen should be streaked on several media in rubber diaphragm screw cap bottles. If, after incubation, there should be any colonies suspected of being *C. immitis*, then approximately 5 ml. of sterile saline should be injected through the rubber diaphragm while keeping alcohol soaked cotton wrapped around the needle and the bottle cap. If the culture is contaminated with other microorganisms, penicillin and streptomycin should be added to the saline. With the alcohol soaked cotton held around the cap, the bottle should be shaken vigorously. The suspension can be withdrawn being careful to hold the wad of cotton tightly around the syringe needle which is removed very slowly. Five tenths milliliter of the suspension can be injected, intraperitoneally, into each of several mice and the remainder can be injected through the rubber diaphragm of a bottle of fresh media. The original culture can be sterilized by injecting formaldehyde (0.5 ml. of 8 per cent, or greater, concentration) and incubating for 24 hours at 37° C., at which time the bottle can be opened safely for making slide preparations.

SUMMARY

A technique is described for handling cultures of *Coccidioides immitis* with safety. This consists of growing the fungus on media in bottles fitted with a rubber diaphragm screw cap.

Suspensions of fungous spores can be made by injecting fluid through the rubber diaphragm with a needle and syringe, shaking the bottle, and then withdrawing the suspension with a needle and syringe. As a precaution against the formation of aerosols of infectious material, alcohol soaked cotton should be wrapped around the screw cap and the syringe needle during the process.

Cultures in these bottles can be sterilized by the injection of 0.5 ml. of at least 8 per cent formaldehyde followed by incubation at 37° C. After 24 hours the bottles can be opened for making slide preparations. The microscopic morphology of formaldehyde killed cultures is comparable to that of the viable culture.

ADDENDUM

After the present manuscript had been accepted for publication, it was brought to the attention of the author that the bottle pictured in Fig. 1 is no longer manufactured. Therefore, the technique has been revised to adapt readily available bottles for this use. The screw caps from prescription bottles are soaked in detergent solution until the liners can be removed. A hole, one eighth inch in diameter, is drilled through the center of each cap. Suitable rubber diaphragms are fixed to the inside of the caps with rubber cement and the caps replaced on the bottles.

It has been found advantageous to insert a cotton plugged, sterile, syringe needle through the diaphragm during incubation of cultures. Strains of *Coccidioides immitis* develop aerial mycelium with arthrospores much more profusely under these conditions than when the needle is omitted.

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A HEMODYNAMIC STUDY OF ATRIAL SEPTAL DEFECT AND ASSOCIATED ANOMALIES INVOLVING THE ATRIAL SEPTUM

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REVIEW OF THE LITERATURE

ATRIAL septal defect is one of the commonest congenital anomalies of the heart and one of those more recently amenable to surgical correction. Consequently, in recent years attention has been focused on the hemodynamic characteristics of the anomaly in an effort to learn more of the effect of this defect on the patient, to increase the accuracy of diagnosis, and to provide a more intelligent basis for the selection of candidates for surgical correction.

In uncomplicated atrial septal defects the shunt usually is left to right and less commonly a right to left shunt of significant magnitude coexists.[1]

Two of us (Swan and Wood) and associates[3, 4] have demonstrated that in the usual case of atrial septal defect the blood shunted from left to right is composed predominantly of blood draining from the right lung. Minimal right to left shunting is not uncommon and occurs preferentially from the inferior vena cava[2], the right to left component is usually so small that the oxygen saturation of blood in the systemic arteries is within the range of normal and the patient has no cyanosis.

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The presence of a predominant right-to-left shunt with accompanying cyanosis has been demonstrated only in those patients with atrial septal defects complicated by pulmonary hypertension, cardiac failure, or associated congenital defect.

Because of the left-to-right shunt, the pulmonary blood flow is markedly increased in a patient with uncomplicated atrial septal defect, usually 2 to 3 times the systemic blood flow.[6, 7] The systemic flow has been reported normal or decreased.[6, 8] The pulmonary artery pressure is usually normal or slightly increased.[9] Pulmonary hypertension is relatively uncommon; only 4 of Limón's 50 patients with atrial septal defect had severe pulmonary hypertension.[2] If pulmonary hypertension does occur, it usually does not become evident until early adult life.[2, 10]

PURPOSE

This report is concerned with the basic hemodynamic data in a sufficiently large series of cases to permit substantiation of certain general conclusions with regard to patients with atrial septal defects. Additional features of these lesions, recently unrecognized, are considered in more detail.

Recent surgical progress in the correction of atrial septal defect[11] has necessitated comprehensive classifications[12, 13] of the variation of interatrial communication, for technical methods for the closure of such defects may differ according to the lesion present.

CLASSIFICATION OF INTERATRIAL COMMUNICATION

The following is a classification of interatrial communications that is of practical value.

I. Atrial septal defect
    A. Foramen ovale type
    B. Located cephalad to fossa ovalis and associated with anomalous connections of right pulmonary veins
II. Persistent common atrioventricular canal
    A. Partial
    B. Complete
III. Unusual communications via coronary sinus
IV. Valve-competent foramen ovale

A defect of the valve-competent foramen ovale type (IV) exists in about 25 per cent of normal human beings[12] and does not cause a detectable abnormality unless pressure in the right atrium exceeds that in the left. Defects high in the atrial septum (I., B) which do not involve the fossa ovalis are usually associated with anomalous right pulmonary veins connected to the junction of the superior vena cava and right atrium.[11] A defect in the region of the fossa ovalis may coexist. An anomalous connection of the pulmonary veins may coexist with any of the varieties of interatrial communication.

METHODS

Cardiac catheterization was carried out by the techniques described elsewhere. Seven of the patients, all less than 8 years of age, were anesthetized by means of tribromoethanol

(Avertin) given rectally, supplemented when necessary by intermittent intravenous injections of thiopental sodium The doses of the 2 drugs were adjusted according to the size of the patient[15] The unanesthetized adults received 1 5 gr (0 1 Gm) of seconal and 0 5 gr (0 032 Gm) of codeine orally before the procedure, the doses were reduced according to weight for the unanesthetized children

Intravascular pressure transmitted through the catheter and systemic artery pressures were recorded by a photo oscillographic assembly,[16] using specially adapted strain gauge manometers

When pressures between two locations in the heart and great vessels were to be compared, only those pressures recorded immediately before and just after withdrawal of the catheter from the one site into the other were evaluated

Mean pulmonary and systemic arterial pressures were calculated by adding a third of the pulse pressure to the diastolic pressure The average mean pressure obtained by planimetric integration of the pressure records in 10 patients did not differ significantly from the mean pressure obtained by this method

An oximeter on each ear continuously recorded the oxygen saturation of peripheral arterial blood[17] Samples of blood were drawn through a cuvette oximeter attached to a catheter for intermittent determination of oxygen saturation* at different locations in the heart chambers and great vessels For recognition of left to right shunts, samples were drawn from the various chambers and vessels in rapid succession

Intermittently during the procedure, samples of blood were drawn from the radial artery and chambers of the heart for determination of oxygen saturation by manometric technique[18, 19]

Pulmonary (Q_P) and systemic (Q_S) blood flows† were calculated by the Fick principle from oxygen consumption (V_{O_2}) and oxygen content of samples of blood drawn from the radial (C_{saO_2}) and pulmonary arteries (C_{paO_2}), and venae cavae[20] When the oxygen saturations of blood samples from radial and pulmonary arteries were nearly identical, the blood samples were compared precisely by passing them through the same oximeter By this means differences of saturation of the order of 1 per cent could be measured with accuracy

$$Q_P = \frac{V_{O_2}}{(C_{pvO_2} - C_{paO_2})}, \quad Q_S = \frac{V_{O_2}}{(C_{saO_2} - C_{mvO_2})}$$

Q_P = Pulmonary flow (liters per minute)

Q_S = Systemic flow (liters per minute)

V_O = Oxygen consumption (cubic centimeters per minute)

C_{pvO_2} = Oxygen content (cubic centimeters per liter) of pulmonary vein blood‡

C_{paO_2} = Oxygen content (cubic centimeters per liter) of pulmonary arterial blood

C_{saO} = Oxygen content (cubic centimeters per liter) of systemic arterial blood

$C_{m\ O}$ = Oxygen content (cubic centimeters per liter) of mixed venous blood

$$\frac{C_{svcO_2} + 2C_{ivcO_2}}{3} = C_{mvO_2}$$

Left ventricular stroke volume was calculated to equal $\frac{Q_s}{R}$

R = cardiac rate at time that samples of blood were drawn for estimation of systemic flow

Pulmonary and systemic flow and stroke volume were expressed also in relation to body surface of the individual (pulmonary flow index, systemic flow index, stroke volume index)

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*Values are expressed as a percentage of the oxygen capacity These data could be expressed as volume of oxygen per 100 c c of blood by multiplying by the oxygen capacity

†Symbols modified from Standardization of Definitions and Symbols in Respiratory Physiology[20]

‡Patient breathing room air C_{pvO_2} = (98 per cent of oxygen capacity + 0 3) ×10 Patient breathing 100 per cent oxygen C_{pvO_2} = (100 per cent of oxygen capacity + 2) ×10

Shunts were calculated as follows, and expressed as per cent of either pulmonary or systemic flows:

Venoarterial (right-to-left) shunt expressed as per cent of systemic flow = $\frac{(Q_S - Q_P)}{Q_S} \times 100$

Arteriovenous (left-to-right) shunt expressed as per cent of pulmonary flow = $\frac{(Q_P - Q_S)}{Q_P} \times 100$

In the absence of an estimate of oxygen consumption, shunts were calculated as follows:

Venoarterial shunt expressed as per cent of systemic flow $= \frac{C_{pvO_2} - C_{saO_2}}{C_{pvO_2} - C_{mvO_2}} \times 100$

Arteriovenous shunt expressed as per cent of pulmonary flow $= \frac{C_{paO_2} - C_{mvO_2}}{C_{pvO_2} - C_{mvO_2}} \times 100$

Arterial dye-dilution curves were recorded by means of ear oximeters and simultaneously by a cuvette oximeter attached to a needle in the radial artery[21] following injection of Evans blue dye (T-1824) at various sites in the heart and great vessels.[22]

A venoarterial shunt was demonstrated by an abnormally short appearance time for the dye at the recording site and an abnormal initial deflection of the curve.[23] Prolongation of the disappearance slope of the curve confirmed the presence of an arteriovenous shunt.[24]

In those cases in which a right-to-left shunt was indicated by an abnormal indicator-dilution curve, but in which there was no decrease in oxygen saturation of peripheral arterial blood, the per cent of shunt was determined from the dye-dilution curve by a method described previously.[23]

The pulmonary resistance was calculated as follows:

$\frac{P_{Pm} \times 1{,}332 \times 60}{Q_P} = R_P$, where P_{Pm} is the mean pulmonary arterial pressure in millimeters of mercury measured at the time that the blood samples were drawn for calculation of pulmonary blood flow: 1,332 is the conversion factor from millimeters of mercury to dynes per square centimeter; Q_P is pulmonary blood flow in cubic centimeters per second; R_P is total pulmonary resistance expressed in dynes · second · $cm.^{-5}$.

CRITERIA FOR DIAGNOSIS

A diagnosis of atrial septal defect was made after cardiac catheterization when a left-to-right shunt had been demonstrated between the two atria or if a significant right-to-left shunt was present and a small left-to-right shunt was demonstrated inconstantly. The passage of the catheter from the right atrium to the left atrium without accompanying evidence of shunting was not considered necessarily diagnostic of an abnormal interatrial communication, since it is possible for a catheter to pass through a normal valve-competent *foramen ovale.*

The oxygen saturation of blood in the venae cavae and right atrium was determined by drawing samples in rapid succession through a cuvette oximeter. In an unanesthetized patient, the oxygen saturation of blood in the inferior vena cava is almost always higher than that in the superior vena cava so that whenever possible samples were obtained from both vessels. Variation in oxygen saturation occurs at different levels in the inferior vena cava, so that the tip of the catheter was positioned at the level of the diaphragm to insure as representative a sample as possible.

An arteriovenous shunt was considered present if the oxygen saturation of blood in the right atrium was demonstrated to be 2 or more per cent higher than the average oxygen saturation of the mixed venous blood.

In those cases in which arterial dye-dilution curves were recorded after injection of T-1824, the presence of a left-to-right shunt was verified by an abnormally long disappearance slope of the curve, indicating pulmonary recirculation of dyed blood.

After injection of dye into either cava, a short appearance time of the dye at the recording site with an abnormal initial deflection indicated a venoarterial shunt. Confirmation of the site at which the shunt was occurring was obtained by absence of an abnormal initial deflection when dye was injected into the right ventricle. The dye dilution curve is the most accurate method of ascertaining the presence of a right to left shunt. However, in cases in which a dye dilution curve was not obtained, the finding of a peripheral arterial oxygen saturation of less than 94 per cent with the patient breathing air, or the finding of less than 1 volume per cent of oxygen physically dissolved with the patient breathing 100 per cent oxygen, was considered evidence of a venoarterial shunt.

Conditions which cause arterialization of blood in the right atrium, and thus may be confused with atrial septal defect are: (1) common atrioventricular canal, (2) anomalous connection of a pulmonary vein or veins, (3) aneurysm of the posterior aortic sinus with rupture into the right atrium, (4) ventricular septal defect with insufficiency of the tricuspid valve, (5) ventricular septal defect opening into the right atrium, and (6) communication between coronary sinus and left atrium.

MATERIAL STUDIED

The hemodynamic data of those patients having had cardiac catheterization in our laboratory between December, 1947, and October, 1954, were reviewed. There were 121 cases in which a diagnosis was made of abnormal interatrial communication.

Patients who had associated pulmonary hypertension, anomalous connection of a pulmonary vein or mitral valve disease were included in the series because the atrial septal defect was the primary anomaly. Those whose condition was diagnosed as common atrioventricular canal were included because of the similarity of the hemodynamics of the two conditions. Those with other associated abnormalities were excluded because the interatrial communication was considered a part of a more complex anomaly (Table I).

TABLE I. INCIDENCE OF ANATOMIC DEFECTS IN 121 PATIENTS IN WHOM AN INTERATRIAL COMMUNICATION WAS A HEMODYNAMICALLY SIGNIFICANT LESION

	CASES	PER CENT OF TOTAL
Atrial septal defect, uncomplicated	44	36
Associated pulmonary hypertension	13	11
Associated anomalous pulmonary venous connection	6	5
Associated mitral valve disease	2	<1
Common atrioventricular canal	6	5
Total patients considered in this report	71	59
Other associated abnormalities		
Pulmonary stenosis	26	21
Ventricular septal defect	13	11
Tricuspid atresia	5	4
Ebstein's deformity of the tricuspid valve	3	2
Persistent truncus arteriosus	1	1
Patent ductus arteriosus	1	1
Coarctation of the aorta	1	1
Total	121	100

Twenty patients demonstrated a gradient of pressure across the pulmonary valve greater than 50 mm. of mercury and were excluded because this was considered indisputable evidence of stenosis of the pulmonary valve. An additional 6 patients had a pressure gradient greater than 20 mm. of mercury across the valve and were excluded as probably having organic stenosis of the pulmonary valve; a more detailed discussion of this matter will follow.

In 13 cases a ventricular septal defect was associated, and the catheterization data did not indicate that an atrioventricular type of lesion (persistence of a common atrioventricular canal) was present.

TABLE II. HEMODYNAMIC DATA IN 57 CASES OF ATRIAL SEPTAL DEFECT (CASES ARE ARRANGED IN ORDER OF INCREASING PULMONARY BLOOD FLOW)

CASE	AGE (YR.)	PRESSURE (MM. HG)				BLOOD OXYGEN SATURATION (PER CENT)					ARTERIAL BLOOD FLOW (L./MIN./M.^{2})		SHUNT (PER CENT OF BLOOD FLOW)	
		R.A.	R.V.	P.A.	RADIAL ARTERY	I.V.C.	M.R.A.	S.V.C.	P.A. OR R.V.	RADIAL ARTERY	PULMONARY	SYSTEMIC	RIGHT-TO-LEFT	LEFT-TO-RIGHT
1	24	19/6	122/20	121/69	100/76	55	51	49	50	80	0.8	1.1	25	0
2	38	9/6	85/17	82/49		54	50	48	50	75	1.0	1.8	45	0
3	23	8/3	150/2	150/80	114/66	62	62	59	55	82	1.3	3.3	60	0
4*	54	6/3	84/3	84/34	127/71		55	56	55	83	1.5		35†	
5*	26	6/4	139/10	139/89	113/65	65	66	61	61	78	1.6	2.9	45	0
6	45	6/2	117/8	117/54	112/65	52	67	45	71	83	2.3	2.4	30	45
7	45	5/1	85/3		128/69	72	70	59	67	94	3.2	1.6	0	50
8	26	5/2	§	77/48	106/65	71	74	66	71	95	3.4	2.6	15	20
9‡	42	7/12	22/5	22/10	110/67	65	79	68	80	96	4.4	2.2	0	50
10	38	5/2	29/3	21/6	95/51	76	82	71	85	97	4.9	3.0	0	40
11	51	10/3	52/7	33/17	132/72	61	76	64	76	94	5.0	2.0	0	40
12	33	5/2	56/6	56/23	155/94	66	71	62	72	95	5.5	3.8	0	30
13	20	14/5	44/0	31/12	116/69	77	88	88	87	96	5.5	3.0	0	45
14	14	7/3	24/7	21/16	148/78	79	81	71	87	98	6.4		0	50
15‡	24	6/4	69/7	68/43	104/59	77	88	67	87	93	6.4	3.5	20	45
16	58	11/5	42/9	37/20	148/77	67	82	65	85	97	6.7	2.9	0	55
17	16	10/0	47/5	40/16	119/64		88	78	91	95	6.8	4.3	0	40
18	22	8/6	33/7	32/6	144/80	84	87	82	88	98	6.8	3.7	0	45
19	25	3/1	30/9	29/18	120/72	76	89	81	90	95	6.9	3.7	0	45
20	15	6/3	29/0	21/7	97/53	75	84	72	86	97	7.1	3.8	0	45
21‡	14	21/0	37/5	28/10	123/78	78	90	80	91	98	7.2	3.5	0	50
22	15	7/4	30/6		136/78	79	87	79	89	97	7.6	3.7	0	50
23	31	17/10	48/9	44/17	132/74	77	85	67	86	92	7.7	2.7	25	75
24	52	13/4	53/7	43/15	180/108	73	80	71	84	95	7.8	2.5	0	70
25	12	8/2	37/0	22/5	115/60	79	82	74	83	96	7.8	4.4	0	40
26	24	12/7	35/12	31/8	124/69	81	94	77	92	99	8.0	3.8	0	50
27*	47	23/16	114/9	114/39	101/74	79	87	59	87	97	8.1	2.5	0	70
28	47	9/1	37/3	34/24	113/66		95	80	93	98	8.3	2.8	0	65
29‡	33	9/2	31/2	26/9	94/57	80	87	64	88	97	8.4	3.0	0	60
30‡	46	4/–1	21/4	22/8	114/64		88	65	90	95	8.8	2.6	0	70
31	8	5/10	40/6	36/26	147/48	75	79	69	83	96	8.9	4.0	0	55
32	7	7/3	39/3	27/15	84/51		91	82	89	95	9.0	2.6	0	70
33	18	11/5	54/12			75	92	72	92	97	9.0	2.8	0	70
34	22	11/9	49/11	30/21	108/60	71	89	63	87	95	9.0	3.6	0	60

35	19	7/3	30/4	21/8	120/72	83	86	76	85	97	9.2	4.7	0	50
36	24	10/7	36/9	36/13	134/72		92	69	91	96	9.4	2.5	0	75
37	8	7/1	49/12	31/21			89		90	98	9.7	4.4	0	55
38‡	5	5/2	24/4	20/7	92/57	81	91	75	92	96	9.8	4.2	0	55
39‡	26	6/1	37/2	30/15	135/75	83	89	73	91	96	10.3	4.4	15	55
40‡	19	11/5	31/6	21/9	116/58	84	85	76	93	98	10.6	3.1	0	70
41‡	23	8/3	36/3	32/10	125/79	84	91	70	90	99	10.9	3.9	0	65
42‡	40	7/4	31/0	28/8	100/45	83	89	75	90	98	11.5	3.3	0	70
43‡	13	5/1	28/6	25/10	90/60	80	87	76	91	97	13.1	5.2	0	60
44	38	12/3	36/2	32/6	112/69		89	65	90	96	13.3	2.3	0	80
45‡	30	7/3	27/2	25/9	101/54	88	94	81	94	97	13.3	4.2	5	70
46‡	27	15/7	29/7	29/14	116/65	88	95	68	93	98	13.8	3.0	10	80
47	37	10/5	41/9	28/15	108/62		84	74	88	97	14.4	3.3	0	75
48	13	11/6	44/9	29/16	107/65		89	76	92	95	14.4	2.6	0	80
49	20	11/6	36/11	33/15	115/68	81	87	77	92	95	14.7	1.4	0	70
50	26	7/4	43/5	27/16	107/62		93	76	92	97	14.9		0	75
51	36	6/2	130/5	121/57	130/73		89	81	93	97	16.7	5.6	0	60
52	4	6/2	54/7	41/26	83/54	78	90		90	86	16.9	5.0	0	70
53	45	7/2	34/2	33/9	127/78	86	94	65	93	95	17.4	3.7	0	80
54‡	27	12/6	40/6	35/17		88	94	66	93	99	20.7	5.6	0	75
55‖	3	6/2	28/5	22/10		76	88	73	89					60
56‖	27	26/15	78/8	78/14	112/72	37	44	35	39	75			40	10
57‖	26	10/8	108/13	108/61	109/68	72	74	65	74	86			45	20

*Diagnosis confirmed at necropsy

†Right to left shunt by dye

‡Surgical confirmation of diagnosis

§Tachycardia while catheter was in right ventricle

‖Data on oxygen consumption not obtained

Key to abbreviations in Tables II and III RA = Right atrium RV = Right ventricle PA = Main pulmonary artery IVC = Inferior vena cava MRA = Middle of right atrium SVC = Superior vena cava

TABLE III. HEMODYNAMIC DATA IN 14 PATIENTS WITH ATRIAL SEPTAL DEFECT AND ASSOCIATED ANOMALY (CASES ARRANGED IN ORDER OF INCREASING PULMONARY BLOOD FLOW)

CASE	AGE (YR.)	PRESSURE (MM. HG)				BLOOD OXYGEN SATURATION (PER CENT)					ARTERIAL BLOOD FLOW (L./MIN./M.²)		SHUNT (PER CENT OF BLOOD FLOW)	
		R.A.	R.V.	P.A.	RADIAL ARTERY	I.V.C.	M.R.A.	S.V.C.	P.A. OR R.V.	RADIAL ARTERY	PULMONARY	SYSTEMIC	RIGHT-TO-LEFT	LEFT-TO-RIGHT
Anomalous Connection of Pulmonary Veins														
58	31	6/3	115/2	113/52	122/68	67	69	60	68	87	2.6	2.9	30	25
59*	32	11/5	36/13	29/13	124/62	84	91	93	93	98	6.8	3.4	0	50
60*	23	10/6	28/5	29/18	105/70	73	85	63	87	95	8.4	2.8	0	65
61*	30	25/20	55/21	55/30	116/80	81	89	68	87	96	8.9	4.8	0	45
62*	43	9/3	43/8	32/44	107/61	77	95	66	94	96	11.5	2.4	0	80
63*	22	7/0	41/8	28/12	129/72	85	96	79	95	98	14.2	3.1	0	75
Common Atrioventricular Canal														
64	9	7/3	101/3		95/71	71	71	65	87	89	3.1	3.1	30	25
65	5	7/3	32/7	33/17	91/62	71	73	68	87	96	6.4		10	60
66*	27	12/6	32/5	27/6	106/71	80	68	62	85	93	8.6	2.8	5	65
67	7†	13/8	40/7		85/59	83	86	81	88	93	10.9	4.1	10	60
68	35	7/4	26/4	25/9	115/57	78	82	62	91	94	11.6	3.5	10	70
69‡	27	7/3	142/1	137/66	106/69	63	71	64	72	81		3.7	50	25
Mitral Disease														
70*	37	18/13	107/5	107/18	138/99	54	62	52	61	96	3.0	1.6	0	50
71*	23	22/11	34/5	35/14	72/57	72	84	50	87	92	5.6	1.7	20	70

*Surgical confirmation of diagnosis.
†Dextrocardia.
‡Diagnosis confirmed at necropsy.

Since the diagnosis of mild organic stenosis of the pulmonary valve complicating atrial septal defect is based on somewhat arbitrary rules, and since the hemodynamic criteria for diagnosis of common atrioventricular canal have not been definitely established, it is possible that the selected group of cases of uncomplicated atrial septal defect includes some examples of the two conditions.

This study, then, is concerned with the 71 patients in whom the atrial septal defect was the *primary congenital defect*.

RESULTS

The vital statistics and pertinent hemodynamic data for these patients are given in Tables II and III.

Sex and Age.—Forty-nine patients were females, and 22 were males, a ratio of 2 to 1. The ages ranged from 3 to 58 years with more than half of the patients between 21 and 40 years of age (Fig. 1).

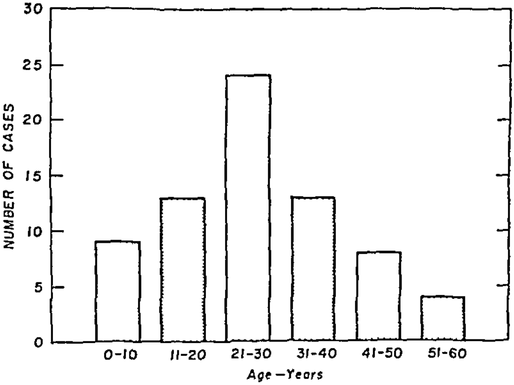

Fig. 1.—Frequency distribution according to age at the time of cardiac catheterization of the 71 patients with interatrial communication considered in this report.

Pulmonary Blood Flow.—The normal cardiac output for adults at rest, breathing air, as calculated by the Fick principle, has been found to average 3.4 liters per minute per square meter (range 2.8 to 5.3 L./min./M.^{2}).[2,3] Based on these values of expected normal pulmonary flow, the series was divided into groups of decreased, normal, moderately increased, and markedly increased pulmonary flow index (Table IV). The blood flow was determined while the patient was at rest and was breathing air, and the mean pulmonary blood flow for the group was 8.3 L./min./M.^{2}, two and a half times normal. In more than three quarters of the group the pulmonary blood flow was moderately to markedly increased. The seven patients with decreased pulmonary blood flow all had severe pulmonary hypertension.

Systemic Blood Flow.—The systemic blood flow at rest was within the range of normal in 69 per cent of the subjects in whom it was measured (Table IV). Only 5 of the group had more than a minimal decrease in systemic flow. All of these had pulmonary hypertension.

TABLE IV. DISTRIBUTION OF PULMONARY AND SYSTEMIC BLOOD FLOWS IN PATIENTS WITH INTERATRIAL COMMUNICATION

	BLOOD FLOW		
	L./MIN./$M.^2$	SYSTEMIC CASES	PULMONARY CASES
Decreased	Less than 2.0	5	7
	2.0-2.8	15	
Normal	2.8-5.3	42	7
Moderate increase	5.4-9.0	3	26
Marked increase	More than 9.0	0	27
Total		65	67

The stroke volume index has been calculated to range between 40 and 65 ml./beat/M.^{2} in normal adults studied in this laboratory while at rest and breathing air. The average for this group was 49 ml./beat/M.^{2} The stroke volume index of the left ventricle was calculated in 21 adults with atrial septal defect in our group who had normal systemic blood flow. The average stroke volume in this group was 44 ml./beat/M.^{2} Sixteen of these patients had stroke volumes between 40 and 60 ml./beat/M.^{2}, which is within the range of normal. The stroke volume of the remaining 5 was somewhat reduced and lay between 30 and 40 ml./beat/M.^{2}; 3 of these had uncomplicated atrial septal defects, 1 had severe pulmonary hypertension, and 1 had common atrioventricular canal. The average left ventricular stroke volume of 8 adult patients with systemic flow of less than 2.8 L./min./M.^{2} was calculated and was found to be 25.1 ml./beat/M.^{2}, with a range from 15.7 to 34.3 ml./beat/M.^{2} Two of the patients with low stroke volumes had uncomplicated atrial septal defect and had a stroke volume that was only minimally reduced, being greater than 30 ml./beat/M.^{2} The remaining 6 were either in cardiac failure or had severe pulmonary hypertension.

There was no correlation between the magnitude of the pulmonary and systemic blood flows in these patients. In patients with uncomplicated atrial septal defect, the systemic blood flow remained within the range of normal despite the magnitude of pulmonary flow.

Oxygen Saturation.—A comparison of oxygen saturation of samples of blood withdrawn in rapid succession from the inferior and superior venae cavae in unanesthetized patients revealed that in most the oxygen saturation was significantly higher in the inferior vena cava than in the superior vena cava (Fig. 2). The average difference was 7 per cent, a difference which is approximately that found in normal adult subjects.

The oxygen saturation of samples of blood drawn in rapid succession from the right ventricle and right atrium differed in many of the subjects (Fig. 3); however, the difference was not constant when sampling was repeated. In 2 patients, the oxygen saturation of samples of right ventricular blood was 6 to 8 per cent higher than that of right atrial blood, suggesting that perhaps a ventricular septal defect coexisted. These 2 patients were operated on for closure of the atrial septal defect, and the ventricular septum in each was found to be intact.

Four of the 6 patients with a diagnosis of common atrioventricular canal showed a consistent and significant increase in the oxygen saturation of blood in the right ventricle over that in the right atrium; the remaining 2 patients had only insignificant increases on repeated sampling.

Shunts.—From the data on blood oxygen saturation it was found that 49 patients (64 per cent) had shunts only in the left-to-right direction while at

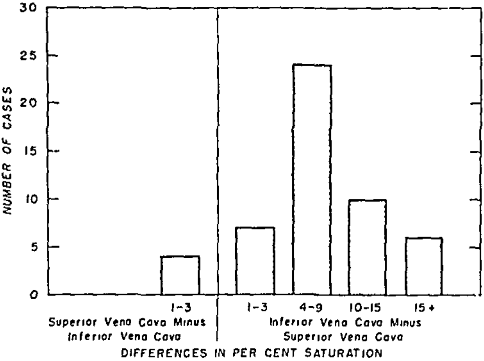

Fig 2—Frequency distribution of differences in oxygen saturation of inferior and superior caval blood in 51 of the patients studied while conscious The determinations were made by means of a cuvette oximeter through which the samples of blood were drawn in quick succession (average interval between samples was 45 seconds) As in normal subjects the saturation of blood from the inferior vena cava exceeds that of superior vena cava by an average of 7 per cent. This relation does not hold under conditions of anesthesia In a few cases the magnitude of this difference suggested that blood in the inferior vena cava was being contaminated by blood of high oxygen saturation either due to an anomalous connection of a pulmonary vein to the inferior vena cava or due to reflux of shunted blood into the inferior cava itself.

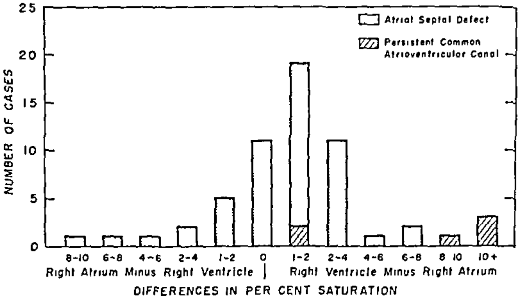

Fig 3—Frequency distribution of differences between the oxygen saturation of blood from the right ventricle and right atrium, in 58 of the patients Samples were obtained successively from just within the right ventricle and from the right atrium close to the tricuspid valve (average interval between samples was ½ minute) Note the wide range of differences which may exist in patients without interventricular communication However, the greatest differences were obtained in 4 of the patients with persistent common atrioventricular canal.

rest and breathing air, 17 (24 per cent) had bidirectional shunts through the defect, and 5 (7 per cent) had right-to-left shunts only. In the more recent catheterizations, small right-to-left shunts from the inferior and superior venae cavae have been demonstrated by the dye-dilution methods in the majority of cases so studied. Only in patients with pulmonary hypertension, however, has the right-to-left shunt been of sufficient magnitude to cause significant arterial desaturation.

More than 70 per cent of patients with right-to-left shunts whether of small or large magnitude showed preferential shunting of blood from the inferior vena cava. More than 70 per cent of those patients in whom dye curves were obtained after injection of dye into the right and left pulmonary arteries showed preferential left-to-right shunting of blood from the right lung. All the patients without pulmonary hypertension showed a left-to-right shunt, which averaged 55 (20 to 70) per cent of the pulmonary blood flow. The right-to-left shunt in these patients, as estimated from the dye-dilution curves, averaged 9 (0 to 20) per cent of the systemic blood flow.

Two of the patients (Cases 27 and 51, Table I) with severe pulmonary hypertension had pure arteriovenous shunts amounting to 70 and 60 per cent of the pulmonary blood flow, respectively.

The 6 patients with common atrioventricular canal all had left-to-right shunts at the atrial level; 2 had demonstrable shunts through both atrial and ventricular septal defects. Two of the 3 patients with dye curves for the right and left pulmonary arteries showed no significant preferential shunting of blood from the right lung. All 6 patients also had right-to-left shunts of 5 to 50 per cent of the systemic flow through the atrial defect, but no right-to-left shunt could be demonstrated through the ventricular defect.

The patient (Case 58) who had an anomalous right pulmonary vein which entered the superior vena cava and severe pulmonary hypertension had a predominant right-to-left shunt.

One patient with mitral stenosis (Case 71) had a large arteriovenous and a small venoarterial shunt through the atrial defect. The other patient with mitral disease (Case 70) had severe mitral insufficiency and an atrial defect of the valve-incompetent foramen ovale type.

Pulmonary Artery Pressure.—Pressure in the pulmonary artery was measured in 66 patients. In 45 it was normal or only minimally increased (systolic pressure 40 mm. of mercury or less). In 6 it was from 41 to 60 mm. of mercury, and the patients were classified as having moderate pulmonary hypertension. Ffteen patients had severe pulmonary hypertension with systolic pressures greater than 60 mm. of mercury (Fig. 4).

Of the 19 patients less than 21 years of age, only one, a child 4½ years of age, had moderate elevation of pressure in the pulmonary artery; the remainder had normal or only minimally elevated pressures. All the other patients with severe hypertension were adults, but only 3 were more than the age of 40 years (Tables II, III, and V).

There was no correlation between pressure and blood flow in the pulmonary arteries of patients who had a mean systolic pressure of 40 mm. of mercury or less (Fig. 5). However, in the group with pulmonary hypertension (mean systolic pressure of more than 40 mm. of mercury in the pulmonary artery)

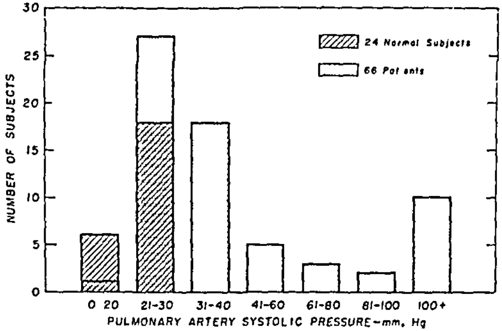

Fig. 4.—Frequency distribution of pulmonary artery systolic pressures in 66 patients with interatrial communication. The data obtained in 24 healthy subjects are included for comparison. Note that many of the patients have pressures similar to or slightly in excess of those obtained in the healthy subjects. Nevertheless, 15 per cent of the patients had pulmonary artery systolic pressures in excess of 100 mm. of mercury.

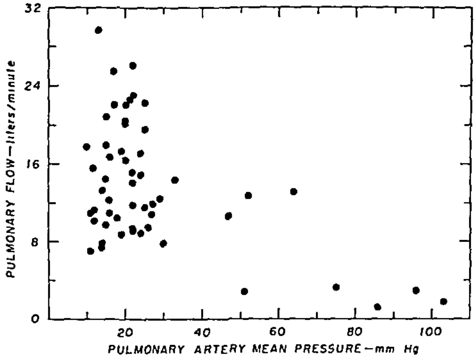

Fig. 5.—Relation of pulmonary blood flow to pulmonary artery mean pressure in 61 patients with interatrial communication. Note the apparent division of the data so that the flow values are almost independent of pressure in those patients with a mean pulmonary artery pressure of less than 30 mm. of mercury. In the patients with a mean pulmonary artery pressure in excess of 40 mm. of mercury there was an inverse relation between pressure and flow.

there was an inverse relation between pulmonary flow and pulmonary artery mean pressure. In Cases 15, 27 and 51 an increased pulmonary blood flow was maintained despite an elevated pulmonary artery mean pressure.

Pressure Gradient Across the Pulmonary Valve.—A significant pressure gradient occurred across the pulmonary valve in many of the patients, so that it was difficult to decide which ones had organic stenosis of the pulmonary

TABLE V. THE RELATION OF PULMONARY ARTERY PRESSURE TO AGE

SYSTOLIC PRESSURE IN PULMONARY ARTERY (MM. HG)	AGE OF PATIENTS IN YEARS			TOTAL	PER CENT
	<21	21-40	>40		
Less than 30	14	12	2	28	42
30 to 40	4	9	4	17	26
41 to 60	1	4	1	6	9
More than 60	0	12	3	15	23
Total	19	37	10	66	100

valve (Fig. 6). However, those patients with a systolic pressure gradient of more than 20 mm. of mercury had normal or low pulmonary blood flow, or a low systolic pressure in the pulmonary artery, and were excluded from the series as probably having organic stenosis of the pulmonary valve. The remaining patients with a significant gradient of pressure across the pulmonary valve had either high pulmonary artery pressure or a pulmonary flow which was greater than normal. Three of the patients with gradients of 10 mm. of mercury or more (Cases 40, 62, and 63) were operated on for closure of the atrial septal defect, and a normal pulmonary valve was demonstrated in each.

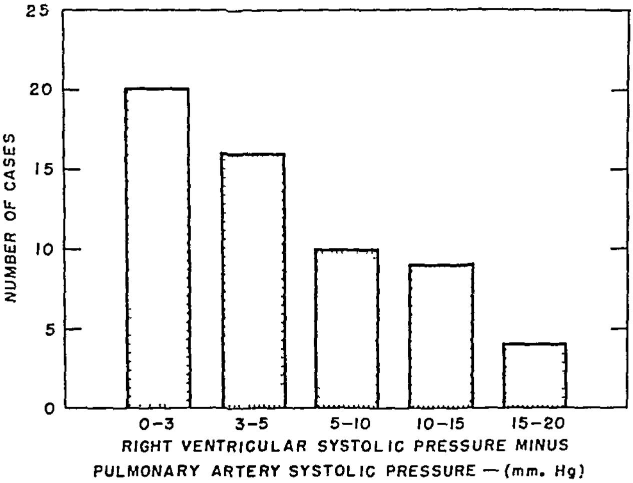

Fig. 6—Frequency distribution of the magnitudes of the systolic pressure gradient across the pulmonary valve in 59 cases of interatrial communication Measurements were only made from records of pressure obtained as the catheter tip was withdrawn across the pulmonary valve and in the absence of cardiac irregularities In healthy subjects the average gradient was 2 mm of mercury (range 0 to 7 mm of mercury)[25]

The magnitude of the gradient was unrelated to the height of the pulmonary artery pressure.

In individual patients there was no positive relationship between pulmonary blood flow and the systolic pressure gradient across the valve; however, there was a poor but statistically significant correlation between pulmonary flows and gradients for the entire group ($r = 0.4$).

Total Pulmonary Resistance.—In those patients without pulmonary hypertension, there was an inverse correlation between pulmonary blood flow and pulmonary resistance. As the pulmonary flow increased, there was a corresponding decrease in calculated pulmonary resistance. In the majority of the subjects with severe pulmonary hypertension, pulmonary blood flow was decreased, and pulmonary vascular resistance was increased considerably.

Common Atrioventricular Canal.—The diagnosis of common atrioventricular canal was made in 6 cases (Cases 64 to 69, Table III). In all, there was a typical roentgenologic appearance of the catheter passing from the right atrium into the left ventricle. In 4 of the 6 there was a significant increase in the oxygen saturation of blood in the right ventricle as compared to that in the right atrium. In one patient (Case 69) who had severe pulmonary hypertension and no left-to-right shunt at ventricular level, the diagnosis of common atrioventricular canal was made at necropsy.

Cleft of the mitral or mitral and tricuspid valves accompanies a persistent common atrioventricular canal. Consequently, the pressure pulses of the right and left atria and the pulmonary artery wedge were examined for abnormally high V waves, which could have resulted from regurgitation of blood through one or both valves. Both of the cases in which the diagnosis was confirmed at operation had normal pressure pulses in the left and right atria.

Lutembacher's Syndrome.—Only one patient (Case 70) was given a diagnosis of mitral stenosis and this diagnosis was confirmed at operation. Of the 23 other patients in the series operated on for closure of the atrial septal defect, the mitral valve was normal in all but one (Case 71) in whom mitral insufficiency was present.

Anomalous Pulmonary Venous Connection.—Six patients had anomalous connection of one or more pulmonary veins. In all but one (Case 58) in whom operation was not performed, the diagnosis was confirmed at surgery.

COMMENT

This study of atrial septal defect and conditions causing nearly identical disturbances of the circulation confirmed certain general impressions Females are more commonly affected than males. The relatively small proportion of children and adolescents studied, which is in contrast to the experience of this laboratory with many other forms of congenital heart disease, supports the impression that many patients enjoy good health during childhood

The evidence is conclusive that in uncomplicated atrial septal defect the pulmonary blood flow is usually at least twice the systemic flow. Despite this substantial increase in pulmonary blood flow the pulmonary artery pressure remains normal or minimally elevated, and calculations of pulmonary vascular resistance give values within or below the range of normal. In adult patients with uncomplicated atrial septal defect, the systemic flow is normal. As the left-to-right shunt increases in magnitude with a consequent increase in pulmonary recirculation, a proportionate decrease in systemic flow might be expected. However, the data show no correlation between the magnitude of pulmonary and systemic flows, and indicate that many patients with uncomplicated atrial septal defect maintain a normal systemic flow and a normal left ventricular stroke volume. Because of the normal difference of oxygen saturation (see below) between blood from the superior vena cava and inferior vena cava, use of the saturation of superior vena caval blood as representative of mixed venous blood for the calculation of systemic blood flow will result in a systematically lower value for systemic blood flow. This may be the explanation for the report of Dexter[26] that the left ventricular output is usually reduced in patients with atrial septal defects.

Certain observations will now be considered in somewhat greater detail.

Oxygen Saturation.—The higher oxygen saturation of blood in the inferior vena cava as compared to the superior vena cava is an important consideration when making the diagnosis of atrial septal defect during cardiac catheterization.

About half of all the blood returning to the right atrium is carried by the inferior vena cava; in normal subjects the oxygen saturation of this blood exceeds that returned by the superior vena cava by an average of 5.7 per cent (range −2 to +16 per cent).[27] Consequently, even in the presence of an intact atrial septum, a sample of blood drawn from the right atrium will usually have a slightly higher oxygen saturation than a sample drawn from the superior vena cava. The certain demonstration of an abnormally high oxygen saturation of right atrial blood is not usually a problem when it exceeds the saturation of superior caval blood by 10 per cent or more. When this is not the case, samples drawn in rapid succession from the inferior vena cava, the right atrium, and the superior vena cava, with the procedure repeated several times if necessary, will frequently enable a decision to be made as to the presence or absence of a left-to-right shunt into the atrium. Anomalous connection of the right pulmonary veins to the superior vena cava with associated interatrial communication has been seen in several patients. In this situation the oxygen saturation of superior caval blood will be equal to or exceed that of right atrial blood, and further samples must be taken upstream from the site of connection to determine the possible nature of such an anomaly. Clearly the demonstration of these differences is greatly facilitated by use of an instantaneous method of high relative accuracy such as the cuvette oximeter for the determination of oxygen saturation. The considerations outlined above are of extreme practical importance in obtaining the highest possible degree of accuracy in diagnosis in the group of patients who are candidates or potential candidates for surgical correction of their abnormalities.

The difference in oxygen saturation demonstrated between the blood in the right ventricle and right atrium is presumably the result of incomplete mixing of blood in the right atrium. The magnitude of such differences, which may frequently be considerable, is evident from Fig. 3. For example, if a sample of blood is drawn from near the coronary sinus and compared with a sample from the right ventricle, the oxygen saturation of blood in the right ventricle will appear greater and a ventricular septal defect might be suspected. Consequently, samples of right atrial blood should be drawn from a position as near to the center of the chamber as possible, and significant increases in oxygen saturation of blood in the right ventricle should be demonstrable in several series of samples drawn in rapid succession from the two chambers before a diagnosis is made of an associated interventricular communication.

Shunts.—The direction of flow across an atrial septal defect is the result of differences in pressure in the right and left atria.[28] In most patients with uncomplicated atrial septal defects, the mean pressure is slightly higher in the left than in the right atrium, although the measurement of such differences is difficult. Such conditions were found in those of our patients in whom left and right atrial pressures were obtained nearly simultaneously.

A plausible explanation for the pressure difference between the atria is the lower filling pressure and greater distensibility of the right than of the left ventricle although Shaffer and associates[28] considered the pressure-volume characteristics of the atria and other factors more important. Although the mean pressure is higher in the left than in the right atrium, at certain times in the cardiac cycle, particularly early in atrial systole and immediately after atrial systole, there appears to be a reversal in gradient for a brief period. This reversal may explain the hemodynamically insignificant right-to-left atrial shunt which is frequently demonstrable by dye dilution techniques.[illegible] The anatomy of the right atrium creates a natural channel for blood flow from the inferior vena cava to the foramen ovale. This may explain the finding that a greater proportion of inferior caval as opposed to superior caval blood shunts right to left in the brief period in which a right-to-left shunt occurs.

Gradient of Pressure Across the Pulmonary Valve.—Many patients with uncomplicated atrial septal defect have lower systolic blood pressure in the pulmonary artery than in the right ventricle. In those of our patients studied after repair of the defect, the pressure gradient was abolished as observed by Blount and co-workers.[29] In the group of 22 healthy subjects studied in this laboratory[25] the average systolic pressure gradient was 2.0 mm. with a range of 0 to 7 mm.

Many factors could be involved in producing the pressure gradient across the pulmonary valve. The relative diameter of the valve, as compared to the size of the right ventricular cavity and the degree of dilatation of the pulmonary artery, could affect the gradient. In the patient with atrial septal defect such a set of circumstances exists, a dilated right ventricle and pulmonary artery are separated by a normal pulmonary valve with resulting "functional pulmonary stenosis."

The height of pressure in the pulmonary artery was not related to the size of the gradient, however, there was a rough correlation between pulmonary blood flow and gradient. As the pulmonary flow increased, the gradient across the valve increased. It may be reasoned that the orifice of the pulmonary valve, although normal in diameter, represents a region of restriction to flow as the narrowest point in the pathway to the lungs. In this location high velocity and turbulence might well be anticipated. Since the systolic murmur of atrial septal defect is heard best over the region of the pulmonary valve, it seems logical to assume that it results from this functional stenosis of the pulmonary valve.

Pulmonary Hypertension.—The incidence of severe pulmonary hypertension is low in atrial septal defect,[9] and it is of interest that none of the patients less than 21 years of age had severe pulmonary hypertension. More recently a child, aged 1½ years, was found to have a pulmonary artery pressure of 54 mm. systolic and 36 mm. diastolic. At operation an atrial septal defect in the usual location was the only anomaly discovered and was repaired successfully. When pulmonary hypertension develops and symptoms become more severe, the patient is more likely to consult a physician and in turn have cardiac catheterization. Consequently, if pulmonary hypertension occurred as commonly in childhood as in later years, these patients would be sent for cardiac catheterization more frequently than those with uncomplicated atrial septal defect. For these reasons, it appears possible that pulmonary hypertension developed as a complication of atrial septal defect in some of the older patients, the duration of the left-to-right shunt possibly being one of the factors in its genesis. Exceptions to this generalization occur, for patients 60 years of age or more have been studied and found to have the clinical and hemodynamic features of atrial septal defect like those usually seen in young adults.

The pulmonary vessels of 2 of the patients in this series with pulmonary hypertension were examined. One (Case 27) had a high systolic pressure in the pulmonary artery and only moderately elevated diastolic pressure; the total pulmonary resistance was only moderately elevated; the pulmonary blood flow was high, and there was a pure left-to-right shunt. Microscopic examination of the lungs revealed medial hypertrophy of the muscular arteries with comparatively few vessels showing intimal changes.

In Case 5, systolic and diastolic pressures in the pulmonary artery were high; the total pulmonary resistance was markedly elevated; the pulmonary blood flow was reduced, and there was a right-to-left shunt through the atrial defect. Microscopic examination revealed extreme intimal thickening of the pulmonary arterioles with occlusion of vessels by connective tissue; there was also medial hypertrophy of the muscular arteries.

Recognition of Other Conditions Which May Simulate Atrial Septal Defect.—

Common atrioventricular canal: The differentiation of common atrioventricular canal at cardiac catheterization is important, since the surgical closure of this complex anomaly is best performed by open cardiotomy with extracorporeal circulation. Satisfactory repair of an atrial septal defect can be accomplished without the use of the latter technique.

Probably the most significant diagnostic finding may be the typical course taken by the catheter when passing from the right atrium into the left ventricle. When this finding is associated with a consistent, significant increase in oxygen saturation of blood in the right ventricle indicating an accompanying ventricular septal defect, there is great likelihood that a common atrioventricular canal is present.

Since the atrial defect in this type of lesion lies in a ventral position in the septum, there is usually a substantial posterior septal rim. Less preferential drainage of blood from the right lung might be expected than in defects lying in the area of the foramen ovale The number of cases studied are not sufficient to allow a conclusion to be drawn, but the indication is that absence of a preferential left-to right shunting of blood from the right lung in the presence of a large pulmonary flow suggests that the interatrial communication may be low and ventral in the septum

Anomalous pulmonary venous connection Care must be taken at cardiac catheterization not to interpret the functional anomalous drainage of the right pulmonary veins frequently found in the usual case of atrial septal defect[3] as evidence for anatomic anomalous connection of the veins from one lung Nor can the apparent passage of a catheter from the region of the right atrium into the right pulmonary veins be taken as certain proof of the presence of an anomalous connection

However, anomalous venous connection of the right lung is associated very frequently with a defect in the atrial septum, and the diagnosis has been made in our cases during catheterization The diagnostic aspects of these conditions have been considered in some detail in a previous publication [4]

Certain cases of complete anomalous pulmonary venous drainage due to connection of all the pulmonary veins to the right atrium or to the superior vena cava with large pulmonary flows may closely simulate the clinical picture of atrial septal defect Data on several such cases have recently been presented from this laboratory [30]

Disease of the Mitral Valve —The diagnosis of mitral stenosis is extremely difficult unless the tip of the catheter enters the left ventricle via the mitral valve It is possible that in addition to the one patient in our series who had mitral stenosis, there might be others in whom the diagnosis was not made The finding of normal pulmonary artery wedge and right atrial or left atrial (if entered) pressures in the presence of normal systemic flow is evidence that a hemodynamically significant degree of mitral stenosis does not exist

If a clinically significant degree of mitral stenosis existed, the pressure level in the atria would be increased If increased atrial pressure associated with reduced systemic blood flow is found in the absence of severe pulmonary hypertension, the possibility of mitral stenosis should be suspected The diagnosis was made preoperatively on this basis in Case 71 reported in this series

In two patients one (Case 27) with and one (Case 21) without pulmonary hypertension, who had very high pressures in the right atria, the mitral and tricuspid valves were found to be normal In Case 21 the systemic flow was 3 5, and in Case 27 it was 2 5 L /min /M ^{2}

It is significant, however, that of the 24 patients of this series operated on, only one (Case 71) had mitral stenosis and no other patient has been found in 45 additional patients studied and operated on since the series was closed The conclusion is that mitral stenosis is an infrequent complication of atrial septal defect

SUMMARY

An interatrial communication was found as a significant lesion in a total of 121 patients studied up to 1954 in the cardiac catheterization laboratory of

the Mayo Clinic. Of these patients, 44 were considered to be examples of uncomplicated atrial septal defect, 13 had associated pulmonary hypertension, 6 had anomalous connection of one or more pulmonary veins, 6 had persistent common atrioventricular canals, and 2 had mitral valve disease. The hemodynamic data obtained from these 71 patients form the basis for this report. In the patients without pulmonary hypertension the pulmonary blood flow was greatly increased (average 8.3 L./min./M.^{2}) due to the presence of left-to-right shunting of considerable degree. In a number of these patients it was demonstrated that of the blood shunted left to right, a greater proportion originated from the right lung than from the left lung. Small right-to-left shunts without hemodynamic significance were shown to exist frequently, and of the blood shunted right to left a greater proportion was of inferior caval than of superior caval origin. In these patients the systemic blood flow was normal. The majority of patients with severe pulmonary hypertension had pulmonary and systemic flows of reduced magnitude. In 2 patients with severe pulmonary hypertension a large pulmonary blood flow was calculated.

Patients with anomalous connection of one or more pulmonary veins or with common atrioventricular canal may show the same fundamental circulatory disorder as patients with an atrial septal defect of the usual type. The frequency and practical importance of each of these lesions are compelling reasons that they be sought and diagnosed preoperatively. Highly suggestive evidence for each of these lesions may be obtained from critical analysis of the oxygen saturation of separate series of samples of blood drawn from different locations in the right ventricle, right atrium and both venae cavae. Differences of some magnitude may exist between samples drawn at random from these sites, but considerable significance may be placed on a difference that is consistently demonstrated.

A significant correlation was demonstrated between the pressure gradient across the pulmonary valve frequently found in these patients and the magnitude of the pulmonary blood flow. The gradient did not appear to be associated with a pathologically significant degree of pulmonary stenosis.

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THE EFFECT OF HEXAMETHONIUM UPON THE PULMONARY VASCULAR RESISTANCE IN MITRAL STENOSIS

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NUMEROUS investigations have been carried out in an effort to establish and delineate a neurogenic control of the pulmonary circulation in man.[1, 2] It has been postulated that there are pulmonary "arterioles" which have a sphincteric action and are under the control of the autonomic nervous system. Their constriction in various pathologic states has been stated to be a factor in the causation of pulmonary hypertension, and the extent that they participate in the regulation of pulmonary arteriolar resistance[3, 4, 14] has been studied by the administration of various autonomic nervous system blocking agents. One such agent is hexamethonium chloride, which is a ganglionic blocking agent and in adequate doses presumably completely inhibits the action of both the sympathetic and parasympathetic nervous systems.[5, 6]

The effect of hexamethonium in lowering the systemic blood pressure has been extensively studied.[7] In addition, its action in patients with pulmonary hypertension has recently come under investigation. Its hypotensive action has been studied in patients with pulmonary emphysema, and in those with heart failure.[8-10] Some authors have come to the conclusion that hexamethonium and similar ganglionic blocking agents have a relaxing effect upon the pulmonary arterioles,[2, 10] while others believe that the lowering of the pulmonary artery and left atrial pressures can be explained by other mechanisms, such as the pooling of blood in the systemic venous circuit and that no direct action of this drug upon the pulmonary vasculature need be postulated.[4, 11-13]

The following report deals with the hemodynamic effects of hexamethonium in patients with pulmonary hypertension, the majority of whom had mitral stenosis. The effects of this drug upon the pulmonary circulation have been studied both in the resting state and during exercise in order to determine in greater detail its effect upon the pulmonary vascular resistance.

METHODS AND MATERIALS

Fifteen patients with pulmonary hypertension were studied by the usual cardiac catheterization technique and in the supine position. Thirteen had mitral stenosis, one had pulmonary hypertension of unknown etiology, and one had lupus erythematosus with

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***Associate Professor of Medicine Director of the Cardiovascular Laboratory, Department of Medicine, University of Colorado School of Medicine pulmonary hypertension The cardiac output, brachial artery, pulmonary artery, and pulmonary artery wedge pressures were determined at rest in all and during exercise in 14 of the 15 patients The patients were in a fasting state and were allowed to rest upon the fluoroscopy table 15 minutes before the start of the procedure The brachial artery needle was then put in place and the pulmonary artery catheterized, and the pressures recorded An additional 10 minutes elapsed before the collection of the expired air and pulmonary and brachial artery blood samples for the determination of the resting cardiac output Pulmonary and brachial artery pressures were recorded at the middle and near the end of this 3 minute period Immediately after termination of the expired air collection, the pulmonary artery pressure was recorded and the pulmonary artery wedge pressure (PAWP) was measured The catheter tip was advanced as far out into the peripheral pulmonary tree as possible, and wedged there While recording the PAWP the catheter tip was withdrawn into the pulmonary artery This maneuver was repeated several times Approximately 5 minutes later exercise was begun after taking control pressure recordings The "warm up" period averaged 2½ minutes and ranged from 2 to 3 minutes, during which time the patients pedaled at constant speed a bicycle attached to the foot of the fluoroscopy table The length of this period and the rate of exercise varied with each patient according to his exercise tolerance A 3 minute expired air collection was then taken for the determination of oxygen consumption Pulmonary and brachial artery blood samples were drawn during the second minute Brachial and pulmonary artery pressures were recorded at 1 minute intervals during exercise A pulmonary artery pressure was recorded and then immediately afterward the PAWP was measured, just after the end of the expired air collection while the patient was still exercising

The patients were then allowed to rest, and pressure recordings continued at intervals until the pulmonary artery pressure had returned to the pre exercise resting level, which usually occurred in 10 to 15 minutes Hexamethonium administration was started 5 to 10 minutes later, after control pressures had been recorded This drug was administered via the cardiac catheter at the rate of 1 mg per minute for the first 10 minutes, the rate was doubled if no fall in systemic pressure had occurred, and if the patient was relatively insensitive it was next given in doses of 5 mg per minute Pressures were measured at 1 minute intervals When the pulmonary artery pressure remained for 4 to 5 minutes at the lowest level that could be attained a resting cardiac output was determined and pressure measurements recorded as above Hexamethonium administration was continued during this period Five of these patients were then immediately exercised, during which time pressure measurements were made, and the cardiac output determined as above The same rate and length of time of exercise were employed in each instance as before hexamethonium had been given The amount of work done was estimated by the amount of oxygen consumed

The average dose of hexamethonium chloride given was 45 mg, and ranged between 15 and 75 mg Side effects were few, and generally consisted of slight blurring of vision, and nausea in a few instances Dizziness and a sharp drop in blood pressure occurred in all patients upon sitting up

The blood oxygen contents were measured by the Van Slyke and Neill technique The oxygen and carbon dioxide of the expired air were determined by the Roughton Scholander method Pressures were recorded with Statham strain gauges and a Hathaway oscillograph

DEFINITIONS

1 The systemic or total peripheral resistance is the resistance offered to blood flow from the left ventricle

$$\text{TPR} = \frac{\text{Mean Brachial Artery Pressure in mm Hg} - 0}{\text{Cardiac Output in c c /sec}} \times 1{,}332$$

2 The total pulmonary resistance is defined as being the resistance offered to blood flow from the right ventricle by the structures in the vascular pathway between the

TABLE I. HEMODYNAMIC DATA OF FIFTEEN PATIENTS WITH PULMONARY

Patient Age B.S.*	Condition	Amount Hex. Admin. (mg.)	Brachial Art. Press. (mm. Hg)			Pul. Art. Press. (mm. Hg)			Pul. Cap. Press. (mm. Hg)	Cardiac Index (L./min. /M.²)	Total Pul. Resist.	Pul. Arteriolar Resist.
			S	D	M	S	D	M				
1. B. J.	Rest		112	62	79	114	58	77	—	2.80	1,545	—
19 1.43	Hex.	15	58	36	43	82	61	68	—	2.67	1,425	—
2. J. G.	Rest		127	73	91	50	26	34	25	2.60	550	146
26 1.93	Hex.	50	87	55	66	40	22	28	19	2.20	541	174
3. J. T.	Rest		120	55	77	85	52	63	24	3.40	984	610
23 1.49	Hex.	100	100	45	67	66	39	48	24	3.10	835	418
4. J. L.	Rest		140	81	101	85	46	59	31	2.53	1,008	479
45 1.84	Hex.	55	110	67	81	63	36	49	27	2.68	794	356
5. L. S.	Rest		125	70	88	82	44	57	32	3.00	887	342
25 1.76	Hex.	50	113	68	83	62	40	47	—	3.30	652	—
6. D. C.	Rest		240	117	158	108	60	76	43	2.49	1,468	638
29 1.66	Hex.	20	119	74	89	72	42	52	25	2.51	995	517
7. M. C.	Rest		134	65	88	71	34	46	—	3.25	546	—
29 2.07	Hex.	55	92	56	68	44	29	34	—	2.25	584	—
8. I. B.	Rest		132	82	98	55	25	35	6	2.49	653	541
44 1.72	Hex.	25	110	71	84	25	13	17	5	2.58	306	217
9. M. D.	Rest		133	73	93	51	24	33	22	2.03	788	265
28 1.65	Hex.	40	102	61	75	39	25	30	25	2.70	541	90
10. H. L.	Rest		185	102	130	126	63	84	16	4.36	944	180
56 1.63	Hex.	50	120	76	91	80	38	52	—	3.64	700	—
K. P.	Rest		117	75	89	94	62	73	23	1.89	1,700	1,167
2 1.82	Hex.	25	103	76	85	80	54	63	21	1.73	1,600	1,067
W. B.	Rest		118	64	81	106	49	68	31	2.90	968	527
3	Hex.	30	98	58	71	54	32	39	15	2.80	581	358
13. G. R.	Rest		133	78	96	40	18	29	18	1.79	611	231
48 2.12	Hex.	50	103	73	83	31	18	22	20	2.68	310	17
14. B. C.	Rest		134	77	96	45	22	30	25	2.99	424	70
34 1.89	Hex.	27	100	60	73	28	17	22	20	3.23	287	26
15. J. S.	Rest		108	62	77	42	17	25	22	2.53	471	56
28 1.89	Hex.	75	100	62	75	38	21	27	16	2.32	551	220

*B.S. = Body surface area in M.²

†Kg. m./min./M.²

For key to abbreviations see footnote to Table II.

pulmonary artery and the left ventricle, and includes the resistance offered by pulmonary vasculature and by the mitral valve. Total pulmonary resistance =

$$\frac{\text{Mean Pulmonary Artery Pressure in mm. Hg - O}}{\text{Cardiac Output in c.c./sec.}} \times 1{,}332$$

3. The pulmonary arteriolar resistance is an estimation of the resistance to blood flow offered by the entire pulmonary vasculature from the pulmonary artery to the left atrium. The so-called "pulmonary capillary" or pulmonary artery wedge pressure (PAWP) is in reality a close approximation to the left atrial pressure, and not the pressure in the capillaries of the lungs. The pressure gradient under consideration, therefore, exists between the pulmonary artery and the left atrium. Pulmonary arteriolar resistance =

$$\frac{\text{Mean Pulmonary Artery Pressure in mm. Hg - PAWP in mm. Hg}}{\text{Cardiac Output in c.c./sec.}} \times 1{,}332$$

Hypertension at Rest Before and After Hexamethonium Administration

Total Periph Resist	Pulse Beats (per min)	Stroke Index (c c /min /M²)	Work R V (Kg m t)	Work L V (Kg m t)	Br Art O₂ (Sat %)	Pul Art O₂ (Sat, %)	O Consump (c c / min)	Diagnosis
1,584	110	25.5	2.9	3.0	93.1	66.1	189	Mitral stenosis
900	115	23.2	2.5	1.6	82.7	55.3	184	
1,472	93	28.0	1.2	3.2	94.1	67.7	272	Mitral stenosis
1,275	111	19.8	0.8	2.0	88.7	60.0	247	
1,200	90	37.8	2.9	3.6	93.2	69.5	218	Mitral stenosis
1,165	90	34.5	2.0	2.1	95.9	68.5	226	
1,728	95	26.7	2.0	3.5	90.2	57.6	282	Mitral stenosis
1,312	125	21.4	1.8	3.0	87.6	77.7	273	
1,370	82	36.6	2.3	3.9	96.0	69.0	246	Mitral stenosis
1,150	120	27.5	2.1	2.7	90.2	61.2	296	
3,052	96	26.0	2.6	5.4	81.7	49.5	247	Mitral stenosis
1,895	73	34.7	1.8	3.0	79.0	52.8	203	
1,044	100	32.5	2.1	3.9	96.2	70.9	303	Mitral stenosis
1,167	120	18.8	1.0	2.1	91.2	60.5	276	
1,380	83	30.0	1.2	3.3	90.9	62.0	229	Lupus erythematosus
1,154	110	23.5	0.6	2.9	95.1	64.7	246	
2,218	85	23.9	0.9	2.6	96.8	64.5	212	Mitral stenosis
1,350	88	30.7	1.1	2.8	93.5	68.6	217	
1,460	100	43.6	5.0	7.7	81.4	67.6	255	Pul hypertension Unknown etiology
1,225	97	37.6	2.6	4.5	86.5	70.0	255	
2,076	84	22.5	1.9	2.3	78.5	51.8	219	Mitral stenosis
2,158	112	15.5	1.5	2.0	84.5	52.2	236	
1,152	116	25.0	2.7	3.2	89.2	60.7	209	Mitral stenosis
1,057	110	25.5	1.5	2.7	84.0	54.3	262	
2,020	55	32.6	0.7	2.3	94.7	67.1	199	Mitral stenosis
1,168	88	30.5	0.8	3.0	95.3	70.7	266	
1,357	77	38.8	1.2	3.9	97.4	72.4	268	Mitral stenosis
957	110	29.4	1.0	3.2	93.2	71.1	255	
1,448	58	43.7	0.9	2.6	91.7	61.3	240	Mitral stenosis
1,530	82	28.3	0.8	2.4	92.4	60.1	241	

All resistances are expressed in absolute units (A U) the dimensions of which are dynes sec cm^{-5}

The significance of the changes in pressure, resistance, and blood flow was evaluated statistically by Student's "t" test, as applied to the absolute values (not the percentage changes)

RESULTS

Resting State—The following hemodynamic changes occurred in 15 patients

Pressures A decrease in the brachial artery pressure occurred in all patients (Tables I and II), and was statistically very significant

Table II. Mean Values in Fifteen Patients With Pulmonary Hypertension at Rest Before and After Hexamethonium Administration

	B.A.P. (mm. Hg)		P.A.P. (mm. Hg)		Pul. Cap. Press. (mm. Hg)	C.I. (L./min./M.^{2})	Total Pul. Resist.	Pul. Arte-riolar Resist.*	Total Periph. Resist.	Ht. R. (beats/min.)	S.I. (c.c./min./M.^{2})	Wk. R.V. (Kg. m./min./M.^{2})	Wk. L.V. (Kg. m./min./M.^{2})	Br. Art. O_2 Sat. (%)	P.A. O_2 Sat. (%)	A-V O_2 Diff. (Vol. %)	O_2 Consump. (c.c./min.)
	$\frac{S}{D}$	M	$\frac{S}{D}$	M													
Before	$\frac{137}{76}$	96	$\frac{80}{40}$	53	24*	2.70	884	430	1,638	88	31.3	2.3	3.7	91.0	63.8	5.22	244
After hex. (45 mg.) av. dose	$\frac{101}{62}$	69	$\frac{54}{32}$	40	20	2.69	713	314	1,298	103	26.7	1.4	2.7	89.3	61.9	5.28	228
Mean per cent change	$\frac{-26}{-18}$	-28	$\frac{-32}{-20}$	-25	-17	0	-19	-27	-21	+15	-15	-39	-27	-1.9	-3.0	-1	-6
Statist. significance	$t = 4.63$ $p = 0.01$		$t = 5.22$ $p = 0.01$		$t = 2.05$ $p =$ sl. < 0.05		$t = 2.33$ $p = 0.05$	$t = 2.08$ $p =$ sl. < 0.05	$t = 3.41$ $p = 0.01$	$t = 3.06$ $p = 0.01$							

*Measured in 11 patients

Key to abbreviations: B.A.P. = brachial artery pressure; P.A.P. = pulmonary artery pressure; Pul. Cap. Press. = pulmonary capillary pressure measured in 11 patients; C.I. = cardiac index; Total Pul. Resist. = total pulmonary resistance; Pul. Arteriolar Resist. = pulmonary arteriolar resistance; Total Periph. Resist. = total peripheral resistance; Ht. R. = heart rate; S.I. = stroke index; Wk. R.V. = work of right ventricle; Wk. L.V. = work of left ventricle; Br. Art. O_2 Sat. = brachial artery oxygen saturation; P.A. O_2 Sat. = pulmonary artery oxygen saturation; A-V O_2 Diff. = A-V oxygen difference; O_2 Consump. = oxygen consumption.

The pulmonary artery pressure decreased in all instances, and the average mean pulmonary artery pressure fell by approximately the same proportion as the average mean brachial artery pressure This decrease was very significant The mean pulmonary artery pressure decreased to one half of the control level in Patient 8 (I B) with but a slight decrease in the systemic blood pressure, but this was the only instance of such a marked differential decrease A few patients were quite sensitive to the hypotensive effect of hexamethonium (Patients 1, 6, and 8) However about 50 mg was necessary to demonstrate a noticeable effect in the majority Others (Patients 3 and 15) showed but a slight reaction to the administration of even 75 mg or more of hexamethonium

The average "pulmonary capillary" (left atrial) pressure decreased by 17 per cent in the 11 patients where measurement was possible However this decrease was barely significant ($t = 2.05$, p slightly less than 0.05) In some instances there was no change (Patients 3 and 8), or a slight increase (Patients 9 and 13), but in the majority a decrease in the "pulmonary capillary" pressure (PAWP) occurred

Cardiac index There was no change in the average cardiac output after the administration of hexamethonium as compared to the control period However in 2 patients (9, 13) there was an increase in cardiac output, while the output decreased in Patients 7 and 10

Resistance The average systemic resistance (TPR) decreased by 21 per cent following the administration of hexamethonium The total pulmonary resistance decreased by approximately the same percentage However, the pulmonary arteriolar resistance decreased by 27 per cent This decrease was barely significant statistically ($t = 2.08$, p slightly less than 0.05)[15] There was no correlation between the extent of decrease in the pulmonary arteriolar resistance and the drop in total pulmonary resistance in the relatively small number of cases in this series

Pulse The average heart rate increased by 15 per cent over the control level which was significant statistically Because of the increase in heart rate the average stroke index decreased after hexamethonium administration

Work load The average work load of the right ventricle decreased to a greater degree than that of the left ventricle An increase in cardiac output and a decrease in pulmonary artery pressure occurred in Patients 9 and 13 while in Patient 15 they both remained constant as compared with the control levels *The work of the left ventricle decreased in all patients*

Blood gas contents and oxygen consumption There was no significant change in either the average pulmonary arterial blood oxygen content or the A V oxygen difference The average oxygen consumption did not change following the administration of hexamethonium

Summary The administration of hexamethonium to these patients at rest and in the supine position resulted in a decrease in the mean brachial artery pressure and an approximately equal percentage decrease in the mean pulmonary artery pressure (Tables I and II) The decrease in left atrial pressure was not significant The cardiac output remained constant The decrease in

TABLE III. HEMODYNAMIC DATA AT REST AND DURING EXERCISE, BOTH

Patient Age B.S.*	Dose Hex.	State	Brachial Art. Press. (mm. Hg)			Pul. Art. Press. (mm. Hg)			Pul. Cap. Press. (mm. Hg)	Cardiac Index (L./min./M.²)	Total Pul. Resist. (A.U.†)
			S	D	M	S	D	M			
K. P. 42 1.82	Control	Rest	117	75	89	94	62	73	23	1.89	1,700
		Ex.	138	87	104	134	66	95	30	2.38	1,750
	Hex.	Rest	103	76	85	80	54	63	21	1.73	1,600
	25 mg.	Ex.	109	77	88	109	74	86	27	2.25	1,678
W. B. 33 1.90	Control	Rest	118	64	81	106	49	68	31	2.90	968
		Ex.	185	85	118	106	74	103	35	3.90	1,106
	Hex.	Rest	98	58	71	54	32	39	15	2.80	581
	30 mg.	Ex.	122	73	89	127	63	84	37	3.30	1,067
G. R. 48 2.12	Control	Rest	133	78	96	40	18	29	18	1.79	611
		Ex.	150	92	111	86	45	59	34	3.27	679
	Hex.	Rest	103	73	83	31	18	22	20	2.68	310
	27 mg. 67 mg.‡	Ex.	123	80	94	63	41	48	30	2.20	824
B. C. 34 1.89	Control	Rest	134	77	96	45	22	30	25	2.99	424
		Ex.	170	95	120	90	50	66	40	4.37	639
	Hex.	Rest	100	60	73	28	17	22	20	3.23	287
	75 mg.	Ex.	100	60	73	58	33	41	34	3.53	492
J. S. 28 1.69	Control	Rest	108	62	77	42	17	25	22	2.53	471
		Ex.	112	60	79	82	38	53	30	3.55	712
	Hex.	Rest	100	62	75	38	21	27	16	2.32	551
	75 mg.	Ex.	103	60	74	55	32	40	27	3.28	577

*B.S. = Body surface area in M.²
†A.U. = Absolute units.
‡40 mg. additional hexamethonium given before cardiac output determined during exercise.
For key to abbreviations see footnote to Table II.

the total peripheral resistance and total pulmonary resistance were of the same magnitude. The percentage decrease in the pulmonary arteriolar resistance was somewhat greater. The pulse rate increased by 15 per cent. The work of the right and left ventricles was appreciably reduced.

Exercise.—Five of the 15 patients were also studied during exercise both before and after the administration of hexamethonium (Tables III and IV).

Exercise prior to hexamethonium administration resulted in an increase in the average mean brachial artery, pulmonary artery, and pulmonary capillary pressures. The cardiac output increased 42 per cent. The total peripheral resistance remained constant, but the total pulmonary and pulmonary arteriolar resistances increased significantly in all 5 instances, the latter by a greater percentage than the former. The increase in the work of the right ventricle was almost double that of the left ventricle.

The effect of exercise upon the circulation was altered following the administration of hexamethonium. There was insignificant increase in the average mean brachial artery pressure during exercise as compared to the resting state. The average mean pulmonary artery pressure again increased markedly with exercise. The percentage increase in the average pulmonary capillary pressure over the resting level was greater following hexamethonium than before it was given. The average cardiac output increased by only 14 per cent,

BEFORE AND AFTER HEXAMETHONIUM CHLORIDE ADMINISTRATION

Pul Arteriolar Resist (A U)	Total Periph Resist (A U)	Pulse (beats/min)	Stroke Index (cc/min/M²)	Work RV (kg m/min/M²)	Work LV (kg m/min/M²)	Brachial Art O_2 Sat	Pulmonary Art O_2 Sat	O_2 Consump (c c/min)
1,167	2,076	84	22.5	1.9	2.3	78.5	51.8	219
1,200	1,917	112	21.2	3.1	3.4	79.1	41.1	39[illegible]
1,067	2,158	112	15.5	1.5	2.0	84.5	53.2	236
1,152	1,717	124	18.2	2.6	2.7	83.9	41.1	419
527	1,152	116	25.0	2.7	3.2	81.2	60.7	261
730	1,267	140	27.8	5.5	6.3	91.7	35.0	709
358	1,057	110	25.5	1.5	2.7	84.0	54.3	262
596	1,130	142	23.3	3.8	4.0	86.7	34.6	550
231	2,020	55	32.6	0.7	2.3	94.7	67.1	199
288	1,298	93	35.2	2.6	4.9	91.6	42.5	648
28	1,168	88	30.5	0.8	3.0	95.3	70.7	266
309	1,613	118	18.6	1.4	2.8	97.0	48.6	625
70	1,357	77	38.8	1.2	3.9	97.4	72.4	268
252	1,163	116	37.7	3.9	7.1	96.0	37.7	916
26	957	110	29.4	1.0	3.2	93.2	71.1	255
84	875	117	30.2	2.0	3.5	94.9	40.6	690
56	1,448	58	43.7	0.9	2.6	91.7	61.3	246
309	1,060	75	47.4	2.6	3.8	90.4	41.1	560
220	1,530	82	28.3	0.8	2.4	92.4	60.1	241
188	1,068	94	34.9	1.8	3.4	88.1	44.9	402

which was significantly less than before hexamethonium was given. The average total peripheral resistance again remained constant. There was still an appreciable and now equal increase in the average total pulmonary and pulmonary arteriolar resistances during exercise following hexamethonium administration. The percentage increase in the average total pulmonary resistance was greater than before hexamethonium. This change was barely significant statistically. The average percentage increase in the pulmonary arteriolar resistance was now about the same as the average percentage increase in the total pulmonary resistance. The pulmonary arteriolar resistance, however, increased in only 3 of the 5 instances, and this increase was not statistically significant. The increase in the right ventricular work caused by exercise was appreciable and was several times greater than the increase in the left ventricular work.

The hemodynamic data obtained during exercise before hexamethonium was given can be compared to the findings after hexamethonium administration (Table IV). The average absolute mean pressure levels reached in the brachial artery and pulmonary artery were less after giving this drug. The left atrial pressure also was lower, but this change was not significant statistically. There was a decreased cardiac output at the time of the greatest hexamethonium effect. The total peripheral resistance remained unchanged. The average total pulmonary resistance did not decrease and the decrease in the pulmonary arteriolar resistance was small. Neither of these two latter

Table IV. Mean Values of Hemodynamic Data in Five Patients With Pulmonary Hypertension in Group II

Conditions	State	B.A.P. (mm. Hg) S/D	B.A.P. (mm. Hg) M	P.A.P. (mm. Hg) S/D	P.A.P. (mm. Hg) M	Pul. Cap. Press. (mm. Hg)	C.I. (L./min./M.²)	Total Pul. Resist. (A.U.*)	Pul. Arteriolar Resist. (A.U.)	Total Periph. Resist. (A.U.)	Ht. Rate (beats/min.)	S.I. (c.c./min./M.²)	Wk. R.V. (Kg. m./min./M.²)	Wk. L.V. (Kg. m./min./M.²)	Br. A. O₂ Sat. (%)	P.A. O₂ Sat. (%)	O₂ Consump. (c.c./min.)
At rest and during exercise, before hex. admin.	Rest	122/71	88	65/36	45	24	2.42	835	410	1,611	78	32.5	1.5	2.9	90.3	62.7	240
	Exercise	151/84	106	100/55	75	34	3.49	977	556	1,541	107	33.9	3.5	5.1	89.8	39.5	645
	Statist.† Significance	$t = 3.32$ $p = 0.05$		$t = 11.9$ $p = 0.01$		$t = 4.27$ $p = 0.05$	$t = 6.3$ $p = 0.01$	$t = 3.73$ $p = 0.05$	$t = 3.40$ $p = 0.05$	$t = 0.09$ N.S.	$t = 7.1$ $p = 0.01$						
	Per cent Change	+24/+18	+17	+54/+53	+67	+42	+42	+15	+36	-4	+37	+4	+133	+76	-0.6	-37	
At rest and during exercise, after hex. admin. (av. dose = 46 mg.)	Rest	101/66	77	46/28	35	19	2.55	666	340	1,374	100	25.8	1.1	2.6	89.9	62.0	252
	Exercise	111/70	84	82/49	60	31	2.91	928	466	1,280	119	25.0	2.3	3.3	90.1	42.0	537
	Statist.† Significance	$t = 1.78$ N.S.		$t = 4.48$ $p = 0.05$		$t = 4.44$ $p = 0.05$	$t = 1.5$ N.S.	$t = 2.4$ $p =$ sl. < 0.05	$t = 1.21$ N.S.	$t = 0.6$ N.S.	$t = 3.61$ $p = 0.05$						
	Per cent Change	+10/+6	+9	+78/+71	+71	+63	+14	+40	+37	+7	+19	+3	+110	+27	+0.7	-32	
During exercise before and after hex. admin. (av. dose = 46 mg.)	Exercise	151/84	106	100/55	75	34	3.49	977	556	1,541	107	33.9	3.5	5.1	89.8	39.5	645
	Exercise + Hex.	111/70	84	82/49	60	31	2.91	928	466	1,280	119	25.0	2.3	3.3	90.1	42.0	537
	Statist.† Significance	$t = 3.16$ $p = 0.05$		$t = 5.24$ $p = 0.01$		$t = 2.12$ N.S.	$t = 2.82$ $p = 0.05$	$t = 0.94$ N.S.	$t = 0.45$ N.S.	$t = 0.57$ N.S.	$t = 2.54$ N.S.						
	Per cent Change	-26/-17	-21	-18/-11	-20	-9	-17	-5	-18	-17	+11	-26	-34	-35	+0.3	+6.0	

*Absolute units. For key to abbreviations see footnote to Table II. †Pertains to the absolute values, and not to the per cent changes.

changes was significant statistically. The right and left ventricular work output decreased during exercise following the administration of hexamethonium by about the same amount (34 per cent).

Summary: Prior to hexamethonium administration, exercise resulted in a slight increase in the brachial artery pressure, and in a considerable increase in the pulmonary artery and left atrial pressures. The cardiac output increased by 42 per cent. The TPR remained constant but there was a definite increase in the total pulmonary resistance (15 per cent) and a greater increase in the pulmonary arteriolar resistance (36 per cent).

Exercise after hexamethonium administration caused an insignificant rise in the brachial artery pressure when compared to this pressure at rest with hexamethonium. There was still a marked increase in the pulmonary artery and left atrial pressures. The cardiac output increased only slightly (14 per cent). The TPR remained constant as before but there was still a considerable, and now equal, increase in the total pulmonary and pulmonary arteriolar resistances (40 per cent). The increase in the work of the right ventricle was much greater than that of the left ventricle, as it also was prior to hexamethonium administration.

DISCUSSION

The administration of hexamethonium to these patients with pulmonary hypertension while at rest and supine resulted in a decrease in the average mean brachial and pulmonary artery pressures that was approximately equal (Table II). The cardiac output remained constant, presumably due in part to the slight increase (15 per cent) in heart rate.

Two physiological mechanisms may have been effective in the lowering of the pulmonary artery pressure. These are: (1) a redistribution of blood, with a shift from the pulmonary to the systemic circuit, (2) the inhibition of pulmonary "arteriolar" vasoconstriction.

The decrease in pulmonary artery pressure may have been due in part to a redistribution of blood with a shift from the pulmonary to the systemic circuit which resulted from the decrease in total peripheral resistance effected by hexamethonium. That this could have occurred has been demonstrated by Werko and associates[11] as well as by Sarnoff and his co-workers.[12, 13] The latter group stated that when the left atrial pressure was high, the removal of only a small volume of blood caused a striking decrease in pulmonary artery pressure, and that peripheral vasodilation and a systemic pooling of blood could account for this result.

Evidence suggesting that the decrease in pulmonary artery pressure also was effected in part by an inhibition of pulmonary "arteriolar" vasoconstriction by hexamethonium resulted from this study. The percentage decreases in total pulmonary and total peripheral resistance were almost identical. However, the percentage decrease in the average pulmonary "arteriolar" resistance was greater (Table II), indicating that hexamethonium resulted in pulmonary "arteriolar" dilatation with a decrease in the pressure gradient between the pulmonary artery and the left atrium. That the pulmonary vasodilatory response was not more striking might have been the result of pathologic changes in the pulmonary vasculature in mitral stenosis which are frequently present, and thus the response of these vessels might have been somewhat impaired. It must also be considered that the autonomic nervous system might assume a more effective role in the causation of increased pulmonary arteriolar resistance only with higher levels of left atrial pressure, and before marked pathologic changes have taken place in the walls of the blood vessels of the lungs.

That pulmonary "arteriolar" vasoconstriction can be a major factor in the etiology of pulmonary hypertension and that hexamethonium does cause pulmonary "arteriolar" vasodilatation was suggested by the results obtained in Patient 8 (I. B.) who had lupus erythematosus. The administration of hexamethonium resulted in a decrease in the pulmonary arterial pressure of over 50 per cent, while the left atrial pressure and the cardiac output remained constant. The fall in systemic blood pressure was slight, minimizing the effect of any possible decrease in the pulmonary blood volume. This striking decrease in pulmonary arteriolar resistance suggested that the relatively small dose of hexamethonium (25 mg.) resulted in an effective inhibition of the autonomic nervous control of the blool vessels of the lungs in this patient with resultant pulmonary vasodilatation. A lung biopsy done soon after the first cardiac catheterization showed thickening of the alveolar septa by a moderate amount of fibrous tissue and an irregular thickening of the arteriolar walls with some hyalinization of the intima. Despite these pathologic changes, a comparable decrease in pulmonary arteriolar resistance occurred again after giving 25 mg. of hexamethonium at the time of a second study 6 weeks later.

The decrease in the resistance of the pulmonary vasculature (pulmonary arteriolar resistance) following the administration of hexamethonium at rest suggests that a decrease in the neurogenic arteriolar tone occurred. A decrease in the pulmonary arteriolar resistance in patients with mitral stenosis has also been demonstrated by Fowler[2] and by Scott[14] employing tetraethyl ammonium chloride and by Judson and co-workers[4] using hexamethonium. The latter came to the final conclusion that hexamethonium causes changes in the pulmonary circulation secondary to its systemic effects. However, Halmâgyi[18] attributed decreases in pulmonary vascular resistance in patients with mitral stenosis and left heart failure to the inhibition of neurogenic vasoconstrictor tone.

The average cardiac output during exercise was less after hexamethonium administration than during exercise prior to giving this drug (Table IV). Therefore, although the systemic and pulmonary artery pressures had decreased, the systemic, total pulmonary, and pulmonary arteriolar resistances were not significantly altered. A good portion of the small decrease in right ventricular work during exercise was also apparently due to the decreased cardiac output after hexamethonium administration. This finding would limit the usefulness of this drug in chronic pulmonary hypertension.[9] It still is worthy of trial in acute pulmonary hypertension when the patient is at rest and supine, as in pulmonary embolism and acute pulmonary edema. Under these conditions the decrease in total pulmonary and pulmonary arteriolar resistance observed after hexamethonium administration would be desirable.

It is apparent that the increase in pulmonary arteriolar resistance with exercise is not neurogenically mediated since it is approximately equal before and after hexamethonium block. Prior to giving hexamethonium, exercise resulted in an increase of cardiac output of 42 per cent as compared to that present during the resting state. Following the administration of this drug exercise resulted in only a 14 per cent increase in the cardiac output (venous return). This relative decrease in venous return during exercise (Tables III and IV) was presumably due to the pooling of blood in the systemic circuit.[8] Yet exercise after hexamethonium resulted in a considerable elevation of the average pulmonary artery and left atrial pressures in all 5 patients to a level comparable to that present during exercise prior to giving this drug. Therefore, a considerable and almost equal increase in total pulmonary and pulmonary arteriolar resistances occurred. The factors and mechanisms effecting this increase are not entirely clear. It may be hypothesized that a shifting of blood from the pulmonary to the systemic circuit had taken place in the resting state after hexamethonium had been given, resulting in a decreased cross-sectional area of the pulmonary vascular bed and perhaps even a decrease in size of the left atrium.[12, 13] Resistance to flow may vary inversely with the level of pressure due to larger vessel diameters at higher pressures than at lower ones.[16] It might also be postulated that a "critical closing pressure" exists for the pulmonary vessels so that, when the mean pulmonary artery pressure is lowered to a level near the left atrial pressure, a decrease occurs in the number of parallel vascular pathways through the lungs which are perfused with blood. Evidence in favor of this hypothesis has been presented recently.[17] These mechanisms could have resulted in an increased resistance to the slight but sudden increase in blood flow which occurred during exercise after hexamethonium.

SUMMARY AND CONCLUSIONS

1. The administration of hexamethonium chloride via cardiac catheter to 15 patients with pulmonary hypertension at rest and supine resulted in a lowering of the brachial artery, pulmonary artery, and left atrial pressures; the cardiac output remained constant.

2. The pulmonary arteriolar resistance decreased to a greater extent than either the total pulmonary or total peripheral resistances, suggesting the inhibition of pulmonary arteriolar vasoconstriction by hexamethonium.

3. A second factor which may have been effective in lowering the pulmonary arterial pressure was a shifting of blood from the pulmonary to the systemic circuit as a result of the decrease in the total peripheral resistance.

4. The marked increase in pulmonary artery pressure during exercise after hexamethonium administration may be related to the decreased volume

and relative inelasticity of the pulmonary vascular bed but also to a closure of vascular pathways in the lungs, resulting in a greater resistance.

5. The decrease in cardiac output during exercise after hexamethonium administration as compared to the level before the giving of this drug would appear to limit its use in chronic pulmonary hypertension. It might be worthy of trial in acute pulmonary hypertension.

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EVALUATION OF MECAMYLAMINE IN THE TREATMENT OF HYPERTENSION

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ALTHOUGH ganglionic blocking agents have a definite field of usefulness in the management of patients having severe hypertension, their application has been limited by the side effects due to parasympathetic blockade,[1] by their variable absorption by the oral route,[2] and by the depressing effect on general well-being which is apparently induced by their primary effect of producing an orthostatic blood pressure reduction; the latter being chiefly dependent on a reduction in cardiac output.[3, 4] The advent of mecamylamine, a ganglionic blocking agent which does not have the quaternary amine structure and which is totally absorbed by the oral route, led us to study this agent in order to evaluate its effectiveness, acceptability, and important side effects or complications, and to compare its hemodynamic effects with those of other clinically useful ganglionic blocking agents

METHODS

Every patient who remained on mecamylamine therapy more than 3 months has been included in this study. There was a total of 31 patients. Treatment was usually instituted at a dosage of 2.5 mg. the first day, 2.5 mg. twice on the second day, and 2.5 mg 3 times on the third day. Gradual dose increments of 2.5 mg 2 or 3 times daily were given during the following days or weeks until the standing blood pressure recorded in the clinic or by the patient at home fell to 150/90 mm. Hg or until the patient could not tolerate the side effects due to parasympathetic blockade The total daily sustaining dose, which was reached 2 to 4 weeks after starting therapy, usually ranged between 10 and 90 mg, preferably divided into 3 daily doses The average daily dose varied between 25 and 30 mg

Sensitivity to the depressor effects of the medication was essentially unpredictable Because it was seen in one splanchnicectomized and in one uremic patient early in the study, the starting dose was reduced in similar patients to 1 mg twice daily. However, in 4 of 7 sympathectomized subjects the maintenance dose was less than the average; in 3 of the 4 azotemic subjects (NPN 45 to 60 mg per cent) the daily dose exceeded the average

Home blood pressures were taken in the standing position before the 8 A.M. and 6 P.M. dose of medication No other hypotensive agent was used The patients were ambu

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latory and ranged in age from 26 to 56. Treatment with mecamylamine was not instituted unless the diastolic blood pressure as determined in the clinic after 30 minutes' rest was higher than 110 mm. Hg. Only 3 patients had pretreatment systolic pressures below 200 mm. Hg.

RESULTS

Effect on Standing Blood Pressure.—Fig. 1 presents the response of the standing blood pressure to mecamylamine administration. The individual blood pressure means of the first 2 weeks of treatment were chosen because the number of readings prior to treatment was not sufficient in all cases for purposes of comparison. Each mean was calculated by averaging the daily morning and evening blood pressure values recorded at home. It can be seen that 50 per cent of the patients exhibited a significant drop of the standing blood pressure. Fig. 2 shows the distribution of the blood pressure response during therapy in 20 patients having complete home records throughout the 3 months of observation. The classifications of response to therapy were done for each individual and for each week separately. Using this method of evaluation, some individuals changed their degree of response from one week to the other.

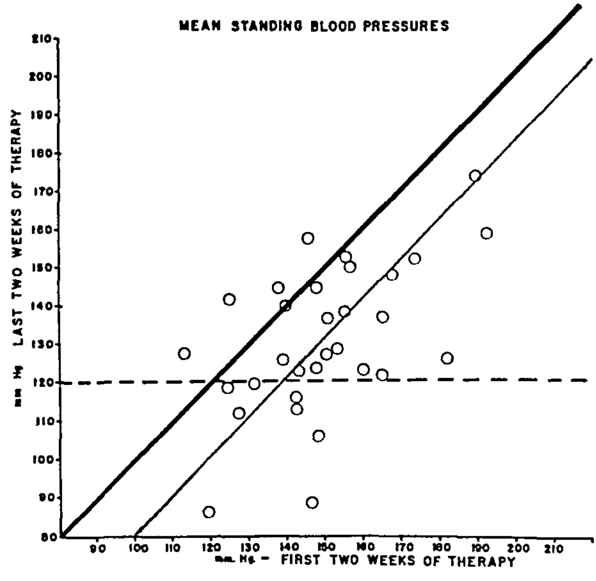

Fig. 1.—The mean values of standing blood pressure during the first 2 weeks of therapy are plotted against those during the last 2 weeks of therapy for each patient. The horizontal interrupted line represents an arbitrary upper limit of mean normal pressure on the scale of the last 2 weeks of therapy. Points lying on the diagonal heavy line represent no change in mean blood pressure, above the line an increase, and below the line a decrease with therapy. Points below the thin diagonal line represent reductions of mean standing blood pressure in excess of 20 mm. Hg.

It is evident from Fig. 2 that the number of individuals showing good and fair responses increased during the first 4 weeks from 35 to 59 per cent. This increment grossly paralleled the increase of the mean mecamylamine dose. With a relatively constant mean maintenance dose ranging from 164 to 172 mg. per week, the per cent of individuals having fair and good responses fluctuated between 59 and 68 per cent between the fifth and eighth weeks.

The per cent showing a good response during this period ranged between 18 and 33. The decrease in this per cent having fair and good responses to 44 during the ninth and tenth weeks of treatment is hardly significant. The mean weekly dose had to be gradually increased to 200 mg. in the twelfth week in order to reach the previous percentage of good and fair responses.

Fig. 2 also demonstrates that a fair or good response had occurred in some patients during the first 2 weeks of therapy. Therefore, the blood pressure means of the first 2 weeks, which were compared in Fig. 1 with the blood pressure means of a later stage of treatment, are not true controls as there has already been some lowering of the blood pressure at this time. Utilizing these criteria reduces the number showing good and fair responses. After the first 4 weeks of therapy the incidence of good or fair responses was not related to the dosage of mecamylamine. This may have been in part the result of inability to increase dosage in some individuals because of side effects.

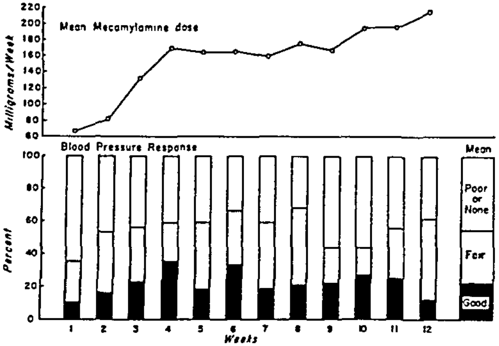

Fig. 2.—The per cent of patients showing a fall in standing blood pressure (below) and the mean dose of mecamylamine administered each week during therapy (above). ■ represents a good response with the standing blood pressure below 150/90 mm. Hg. ▨ represents a fair response with the fall in the mean standing blood pressure being greater than 20 per cent of the mean control pressure but the levels being above 150/90 mm. Hg. □ represents less than 20 per cent reduction from the mean control value.

More careful analysis of apparent "failures" indicates that many are a result of the criteria used. In some of the 9 cases in Fig. 1 having the poorest results, other considerations entered into the failure to reduce the standing blood pressure. In 3 cases there were no home blood pressure records. For example, in the case of D. Ro. a small dose of 4.5 mg. per day was given and appeared effective in reducing the mean blood pressure in the clinic from 145 to 117. Subsequently the clinic blood pressure rose to 130, 151, and 148, but the dose was not increased because of the complaint of orthostatic dizziness at home. In 3 cases use of the blood pressure during the first week of therapy resulted in "control" readings which already reflected therapeutic effect. For example, J. Ar. was admitted to the hospital because of apparent focal encephalopathic episodes and responded immediately to 5 mg. daily. His "control" blood pressure for the first 2 weeks was 122. When the cause of his encephalopathy was found to be unrelated to hypertension, a final treatment blood pressure of 138/100 (119 mean) was considered satisfactory, this being a 50 mm. systolic decrement from the recumbent level. There were 3 clear failures: In one drug induced reductions in pressure caused chest pain; one patient was miserable from gastrointestinal side effects; and in the last patient blood pressure was so variable that sympathectomy was recommended.

Perhaps the degree of blood pressure control achieved can best be demonstrated by the effect of drug withdrawal. Eight patients under satisfactory control were asked to withhold treatment for a period of 1 to 2 weeks. Table I presents these observations. It may be seen that a significant depressor effect was evident in all cases and that the effective dosage had not greatly changed when control was re-established. This indicates lack of tolerance to the medication, since in comparable experiments with quaternary blocking agents a considerable sensitivity to this dosage would have been manifest after such a period of time off therapy.

TABLE I. EFFECT OF TREATMENT WITHDRAWAL ON STANDING BLOOD PRESSURE

PATIENT	STANDING BLOOD PRESSURE (MM. Hg) RELATED TO TREATMENT WITHDRAWAL			DRUG DOSE (MG. PER DAY)	
	BEFORE	DURING	AFTER	BEFORE	AFTER
D. W.	144/ 98	166/122	146/106	60	60
A. C.	140/104*	201/143*	164/128	50	50
W. S.	166/118	215/158	132/ 98	75	60
E. R.	120/ 85*	180/132*	100/ 70*	30	35
L. M.	128/ 90	205/134	152/ 94	45	36
P. R.	125/ 98	250/160	150/115	67	75
L. T.	----	178/128	179/ 95	60	60
L. W.	148/ 92	170/130	----	30	--

*Indicates home blood pressure. All readings otherwise represent the average of one or two casual readings in the clinic on the final day of the period indicated.

Effect on Recumbent Blood Pressure.—Except for one patient among the 31 studied, the effect of mecamylamine on the recumbent blood pressure was negligible.

Discrepancy Between Home and Office Blood Pressure Readings.—It was of interest to compare home and office readings for 25 patients. The mean of the standing blood pressure values recorded the day prior to the clinic visit was compared with the mean clinic standing blood pressure. In 4 instances the home readings significantly exceeded the office readings. In 8 cases the clinic values were significantly higher than the home values and in 13 cases home and office readings did not differ. The reliability of the patient's blood pressure readings was tested by a nurse with the patient taking the blood pressure at the same time and under identical conditions. These showed a satisfactory correspondence. These data confirm the validity of utilizing blood pressure recordings taken in the home in the management of patients having hypertension.[5, 6]

Effects on Renal Function.—Inulin and para-aminohippurate clearance studies with an indwelling bladder catheter were performed in the morning on patients in the recumbent position. Three weeks prior to the clearance the patients were placed on a low salt intake, consisting of a diet to which no salt was added at the table or in the kitchen Two grams of sodium chloride were added to this regime daily by the patient The data presented are the mean of 3 periods, each with a length of fifteen minutes (Table II) It will be seen that, in general, treatment slightly decreased filtration fraction, para aminohippurate and inulin clearance in about half of the individuals studied Usually such changes were associated with a decrease in urine flow and in sodium and chloride clearance, but no distinct change in potassium excretion or nonprotein nitrogen

These changes were then analyzed with respect to the success of antihypertensive therapy in the interval between the two tests Three patients (W L, T L and S W) whose home standing blood pressures did not change significantly showed a decrease in glomerular filtration rate ranging from 21 to 24 per cent Three patients (C A, R E, and M L) having a marked drop of standing blood pressure for a prolonged period of time showed no change in renal plasma flow The glomerular filtration rate decreased in two patients and remained unchanged in one It is interesting to note that these patients who had a significant response to therapy had impairment of their renal function prior to treatment

Effects on Cardiac Output and Cardiac Status—Symptoms of left ventricular failure, which occurred in 8 of the 31 patients, were relieved during the period of study Angina pectoris became worse in 4 and disappeared in 1 individual In our further experience to date with 80 patients treated for variable periods of time mecamylamine did not appear to reduce the incidence of angina or myocardial infarction Indeed in one patient whose standing blood pressure dropped to normal values during treatment with mecamylamine, an anteroseptal myocardial infarction occurred In one other case a lethal myocardial infarction occurred one week after the hypotensive therapy was stopped A third patient exhibited electrocardiographic changes in the course of therapy which were suggestive of a subendocardial infarction In a fourth patient (W W) similar electrocardiographic changes appeared after 4 days of therapy On the basis of these data, it cannot be decided whether this relatively high incidence of myocardial infarction (5 per cent) seen in the total number of treated patients (80) is coincidence or whether mecamylamine did more in promoting coronary occlusion than would any hypotensive agent

The lesser frequency of extreme weakness and fatigue with this drug suggested that perhaps it did not decrease cardiac output as other ganglionic blocking agents have been shown to do[3, 4] Accordingly, through use of the tracers radioactive iodinated serum albumin, or fluorescein, cardiac output and stroke volumes were measured in the sitting position before and during therapy with mecamylamine in 3 individuals One patient who received an effective dose of 60 mg on the second and third days showed a marked decrease (45 per cent) of the cardiac output on the third day However, the high initial cardiac output in this patient was perhaps the result of anxiety and the subsequent reduction might not be entirely attributable to drug

TABLE II. RENAL FUNCTION STUDIES BEFORE AND DURING MECAMYLAMINE THERAPY

PATIENT	TREATMENT DURATION (DAYS)	ARTERIAL BLOOD PRESSURE (MM. HG) RECUMBENT	STANDING	C_{In} (ML./MIN.)	C_{PAH} (ML./MIN.)	FILTRATION FRACTION	URINE VOLUME (ML./MIN.)	C_{Na} (MEQ./MIN.)	C_{Cl} (MEQ./MIN.)	C_K (MEQ./MIN.)	BLOOD NPN (MG. %)
M. L.	77	216/122	224/132	46	124	0.357	1.81	1.3	4.5	12.5	73
		245/128	117/ 81	26	119	0.222	3.55	3.2	3.6	12.9	65
S. D.	59	175/115	182/132	75	367	0.205	5.45	6.2	7.0	33.1	--
		150/112	146/112	77	366	0.210	3.09	3.0	3.5	20.1	--
C. A.	49	215/128	236/154	93	364	0.257	5.80	4.7	5.3	37.3	40
		213/135	142/100	68	343	0.198	1.10	0.7	2.4	11.3	--
W. L.	126	160/120	150/120	138	575	0.240	3.78	4.9	5.4	18.0	30
		146/105	129/101	107	522	0.205	2.50	3.3	4.2	30.0	--
S. W.	86	200/136	202/142	85	359	0.239	4.75	4.3	9.7	20.2	35
		187/124	152/116	65	305	0.213	1.43	2.5	3.2	21.0	38
R. E.	71	190/125	160/130	44	196	0.225	1.49	0.6	4.0	14.0	48
		169/115	118/ 78	47	195	0.241	1.23	0.7	0.7	10.1	37
T. L.	162	185/111	184/112	117	558	0.210	4.20	3.4	3.8	44.7	37
		172/108	149/ 97	93	343	0.270	1.75	0.9	3.5	25.1	41
W. D.	81	232/136	234/144	65	266	0.245	2.93	2.4	2.2	20.0	38
		219/132	158/112	70	261	0.268	4.82	1.7	1.6	18.8	--
R. P.	80	229/159	230/140	60	250	0.241	1.34	1.4	1.4	4.5	35
		198/149	168/132	--	201	----	1.58	1.2	1.2	7.1	46

For each patient the upper row of values was obtained before and the lower row during mecamylamine therapy.

therapy. Two other patients received smaller doses and their sitting blood pressures as well as their cardiac outputs on the seventh and eighth days, respectively, of treatment did not change significantly.

Effect on Blood Coagulation.—Because of the occurrence of coronary thrombosis during therapy a study of blood coagulation factors was undertaken. Studies of prothrombin time, fibrinogen, and factors V and VII revealed no changes in 5 patients studied over a period up to 8 months in duration.[7]

Effect on Number of White Blood Cells and Hemoglobin Concentration.—There were no alterations found over a treatment interval of 2 to 4 months in 10 patients studied.

Side Effects and Complications of Treatment.—The usual effects of parasympathetic blockade were observed (blurred vision, dryness of the mouth, constipation, and impotence) in most patients who received an effective dose. Constipation was the most prominent side effect. Patients who had been on chlorisondamine often noted fewer ocular symptoms when their blood pressure had been reduced to equivalent levels by mecamylamine. The majority of patients reported fewer attacks of dizziness or weakness than when taking pentolinium. Constipation, dry mouth and impotence were as frequently noted with mecamylamine as with other ganglioplegic drugs.

Ten of the patients complained of some loss of muscular strength. One patient developed ileus and volvulus while on a small therapeutic dose of 5 mg. daily. An unusual side effect noted by 2 patients was extreme chilliness associated with a tremor. A confused mental state developed in 2 individuals. Such production of tremor and mental confusion has been noticed as well by others. This is sometimes related to impaired renal function, although as noted above we have treated 4 chronically azotemic individuals without difficulty. In all instances the effects have cleared on withdrawing therapy. In Table III are summarized the side effects among the present consecutive series and in our subsequent experience to date (80 cases in all).

Effects on the Retinal and Cerebral Circulation.—In a few patients fresh retinal hemorrhages and exudates appeared to clear during prolonged therapy with mecamylamine. Cerebrovascular episodes have not recurred during the 60 patient months of treatment in 5 subjects who before treatment had had a cerebrovascular accident. Unpublished observations[8] would indicate that 30 per cent of the hypertensive patients suffering one focal neurologic episode will have a recurrence within 5 years. Our experience is, therefore, inadequate to draw satisfactory conclusions concerning the prevention of this complication.

DISCUSSION

Mecamylamine has proved to be a useful ganglionic blocking drug for the management of severe hypertension. The frequency of orthostatic blood pressure reduction achieved in this series of 31 patients over a 3 month interval is comparable to that observed with quaternary blocking agents. Twenty-three per cent of the patients showed a good blood pressure response their

TABLE III. REASONS FOR ADVISING CESSATION OF MECAMYLAMINE THERAPY

			UNACCEPTABLE SIDE EFFECTS	
CASE INITIALS	TREATMENT DURATION	TOTAL DAILY DOSE (MG.)	INTRACTABLE GI SIDE EFFECTS	OTHER CAUSES
1. E. Kl.	2 mo.	20		Also had adequate control on reserpine alone
2. M. Cl.	2 mo.	40	Yes	Poor blood pressure response
3. C. Ro.	4 mo.	50	Yes	Poor blood pressure control
4. M. Po.	4 mo.	25	Yes	Poor blood pressure control
5. K. La.	3 mo.	30	Yes	Poor blood pressure control
6. F. Ro.*	2 mo.	6	Yes	Increased angina at same blood pressure reduction as achieved by other ganglion blockers
7. C. Br.	5 mo.	45	Yes	Constant anterior chest pain. Poor blood pressure control
8. E. Tu.	1½ mo.	30	Yes	
9. M. Bu.	2 days	2.5	Yes	60 year old woman suddenly developed a marked ileus; at operation volvulus found
10. F. Fa.*	3 days	5		Cerebrovascular episode on third day
11. J. Vo.	3 mo.	18		Malignant hypertension with rapid terminal azotemia
12. R. Mo.	2 mo.	35	Yes	Also itching, sore eyelids, "jitters," and dyspnea, recurrent when drug restarted
13. P. Bu.	1 mo.	35		"Jittery," gross tremor, memory loss, and confusion
14. G. Re.*	7 days	3 9		Early uremic symptoms. NPN 113. After 5 days irregular treatment patient developed confusion, aphasia, increased motor activity, and orthopnea. When treatment stopped, he lapsed into uremic stupor

Above cases represent all instances among the last 80 patients placed on mecamylamine treatment in which it has seemed advisable to the clinic physician to omit the drug.

*Indicates coexistence of moderately elevated nonprotein nitrogen

standing blood pressures falling to normal levels. Thirty-three per cent exhibited a fair response, while in 44 per cent there was an unsatisfactory response. Seven patients had had splanchnicectomies prior to treatment and in 4 cases their maintenance doses were less than the average. That sympathectomy sensitizes the patient to ganglionic blocking agents has been demonstrated in the case of tetraethyl ammonium responses.[9]

Each patient must find that dose which effectively lowers his standing blood pressure. Standing blood pressures taken before breakfast and supper are fairly representative of the total diurnal effect. Readings at these times permit adjustment of individual doses to secure better morning or evening blood pressure control. When dizziness or fatigue at noontime is reported, blood pressure should be checked since usually it is lower at this time than in the evening. Medication should be taken at mealtime and increased by 2.5 mg increments every second or third day until the average standing blood pressure reaches normal values or side effects make further increase impossible.

Although the effect on recumbent blood pressure is negligible, definite cardiac and retinal improvement has occurred in some patients. Renal circulation is unimproved or may be considerably reduced in the upright position,[10] and cardiac output appears to be reduced as with other ganglionic blocking agents. Corcoran and Page[11] have shown that renal plasma flow may improve only after years of therapy with hexamethonium so that transient reductions of renal plasma flow in the early stage of treatment should not discourage one from using a ganglionic blocker. One should make sure that the functional renal reserve is sufficient to stand transient reductions of renal blood flow. Repeated checking of the nonprotein nitrogen or blood urea nitrogen is a necessity in cases with azotemia.

Although the relatively high incidence of coronary thrombosis may be coincidental, it is pertinent to stress the importance of this complication. In other series this condition has not increased with effective therapy.[12] Patients having suffered a myocardial infarction or having signs of coronary insufficiency should be treated very carefully with ganglionic blocking agents and marked falls of their standing blood pressures must be avoided.

Side effects were comparable to those with quaternary ammonium compounds. Weakness appeared to be a less frequent complaint with mecamylamine than with other ganglionic blocking agents. Constipating effects were similar to those following other ganglionic blocking drugs but since absorption is relatively complete, variation in bowel motility does not affect the amount absorbed. This is a further advantage of this drug. Visual effects were possibly less marked with mecamylamine. Dry mouth and impotence were about as frequent as with quaternary blocking drugs. Tolerance appeared to be negligible. These observations are in general agreement with those of others investigating this form of therapy.[12, 13]

SUMMARY

1. The status of 31 consecutive hypertensive patients treated as outpatients with mecamylamine and followed for over 3 months was examined. A daily record of standing blood pressure showed a significant drop in over 50 per cent. Approximately one third showed normal daily standing blood pressures. In approximately 20 per cent medication had to be discontinued because of distressing side effects.

2. Chronic administration of the drug produced only a slight reduction in renal plasma flow, glomerular filtration rate, and in sodium clearance in about half the cases.

3. In 2 cases in which there was little change in the sitting blood pressure cardiac output was unaltered. In a third instance a reduction in cardiac output occurred concomitant with a pronounced fall in sitting blood pressure.

TABLE I. EFFECTS OF I.M. ACTH GEL IN 5 NORMAL SUBJECTS (MEANS ± S.D.)

PATIENT	TIME	NO. DAYS	GASTRIC PEPSIN (P.U./HR.)	EOSINOPHILS (CELLS PER C.MM.)	URINE				PLASMA		CLEARANCE (L./24 HR.)	
					17-HYDROXYCORTICOID (MG./24 HR.)	VOLUME (L./24 HR.)	CREATININE (GM./24 HR.)	PEPSINOGEN (P.U./HR.)	CREATININE (MG. %)	PEPSINOGEN (P.U. %)	CREATININE	PEPSINOGEN
O. G. W.	Pre-	7	89,000	200 ± 30	0.5 ± 0.8	1.300 ± .283	2.313 ± .125	317 ± 32	1.27 ± .01	14.1 ± 2.4	184 ± 8	57 ± 8
	ACTH	5	86,000	18 ± 12	23.7 ± 12.5	1.030 ± .262	2.400 ± .098	438 ± 41	1.25 ± .08	15.2 ± 4.0	193 ± 5	76 ± 24
	Post	7	56,000	216 ± 28	1.4 ± 2.0	2.030 ± .289	2.436 ± .156	304 ± 30	1.37 ± .07	16.3 ± 2.0	177 ± 7	45 ± 9
D. B.	Pre-	7	40,000	230 ± 26	0.9 ± 2.2	2.430 ± .977	2.376 ± .10	212 ± 30	1.20 ± .05	11.0 ± 1.4	197 ± 14	49 ± 6
	ACTH	5	36,000	2 ± 3	57.3 ± 16.5	1.370 ± .103	2.700 ± .165	424 ± 33	1.11 ± .08	10.4 ± 2.6	242 ± 32	104 ± 30
	Post	9	50,000	291 ± 32	1.5 ± 1.7	3.030 ± .880	2.461 ± .134	205 ± 46	1.29 ± .05	14.9 ± 5.1	195 ± 17	37 ± 15
J. D.	Pre-	8	125,000	146 ± 28	1.3 ± 2.0	1.050 ± .254	1.796 ± .14	208 ± 42	1.16 ± .13	11.5 ± 2.2	144 ± 15	43 ± 11
	ACTH	6	156,000	55 ± 34	13.1 ± 7.4	.906 ± .209	1.867 ± .15	310 ± 33	1.05 ± .04	15.0 ± 1.4	184 ± 9	51 ± 3
	Post	5	112,000	185 ± 17	0.4 ± 0.4	1.960 ± .242	1.950 ± .16	201 ± 54	1.37 ± .07	13.8 ± 0.7	143 ± 7	35 ± 0
A. B.	Pre-	5	66,000	108 ± 48	2.7 ± 0.81	1.930 ± .880	2.382 ± .121	277 ± 34	1.28 ± .12	15.7 ± 1.0	169 ± 3	42 ± 9
	ACTH	6	81,000	15 ± 11	27.8 ± 11.1	1.190 ± .209	2.228 ± .241	160 ± 58	–	15.6 ± 5.0	–	71 ± 16
	Post	4	61,000	84 ± 40	0.2 ± 0.2	2.115 ± .532	2.290 ± .38	272 ± 24	–	15.8 ± 2.7	–	43 ± 8
D. C.	Pre-	5	207,000	224 ± 53	1.4 ± 2.5	1.340 ± .391	1.982 ± .19	340 ± 67	1.24 ± 0	17.7 ± 2.5	147 ± 7	43 ± 7
	ACTH	6	226,000	17 ± 12	13.2 ± 3.0	1.260 ± .242	2.150 ± .18	523 ± 53	–	19.9 ± 2.6	–	64 ± 8
	Post	4	205,000	87 ± 33	0.4 ± 0.2	1.895 ± .601	2.06 ± .09	352 ± 57	–	21.1 ± 1.7	–	40 ± 6

of treatment and remained at that plateau for the 6 days of the ACTH administration There were increases in the clearances of both creatinine and pepsinogen in all the subjects in whom these measurements were made The changes in creatinine clearance were accounted for by changes in both the urinary and plasma creatinine, while the increases in pepsinogen clearances were almost entirely due to changes in the urinary pepsinogen (see Fig 2) The mean values in these 5 subjects for the basal output of gastric pepsin before, during, and after ACTH (Table I) indicate the variable effects of ACTH on gastric pepsin and the independently variable changes in plasma pepsinogen concentration These contrast with the invariable steep increases in urinary pepsinogen excretion due to ACTH Fig 1 shows, furthermore, that there was little if any direct correlation between simultaneous measurements of gastric pepsin and urinary pepsinogen

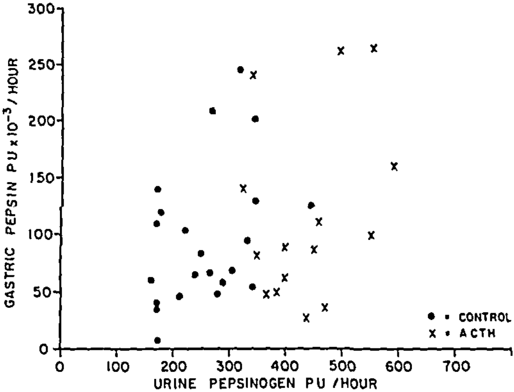

Fig 1—Scatter diagram showing poor correlation between gastric pepsin secretion and simultaneous urinary pepsinogen excretion

Fig 2 shows the correlation between the level of plasma pepsinogen and the pepsinogen in the urine in the control and ACTH samples in these experiments, and includes 29 samples from 6 patients with peptic ulceration, 2 of whom have been reported elsewhere[1 7] The mean clearance of pepsinogen in the control periods in normals was 44 ± 14 liters in 24 hours, in the ulcer subjects, 36 ± 19 liters per 24 hours, and in normal subjects under the influence of ACTH, 65 ± 29 liters per 24 hours, a level significantly higher at the 1 per cent level than either the levels in the control periods or in the ulcer subjects The increase in pepsinogen clearance as stated above resulted from increases in urine pepsinogen with little change in plasma pepsinogen

Simultaneous measurements of pepsinogen and creatinine clearances were obtained in the control periods of all the subjects, but in only 3 subjects during and after ACTH administration, and in one subject (Fig 3, labeled "abnormal patient") during a second ACTH study 3 months after the first, when there had been a marked decline in the clearances of both pepsinogen and creatinine. The correlation of the clearances of these 2 substances is shown in Fig. 3 as

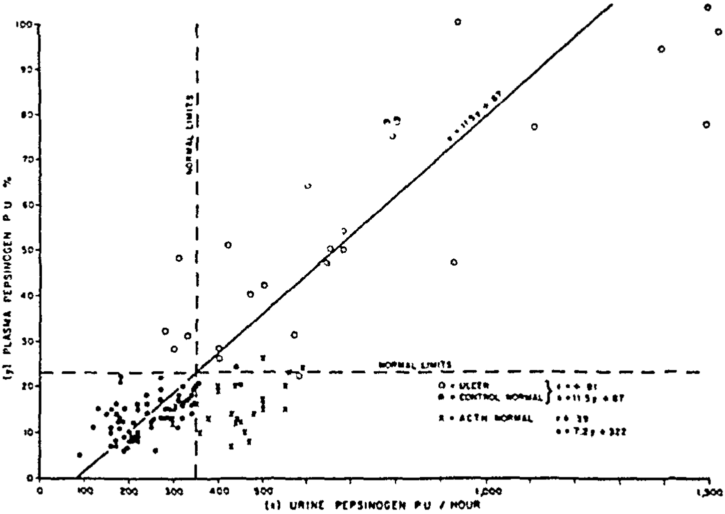

Fig. 2.—Illustration of the different mechanisms responsible for the increased urinary pepsinogen excretion in ulcer patients and in normals given ACTH.

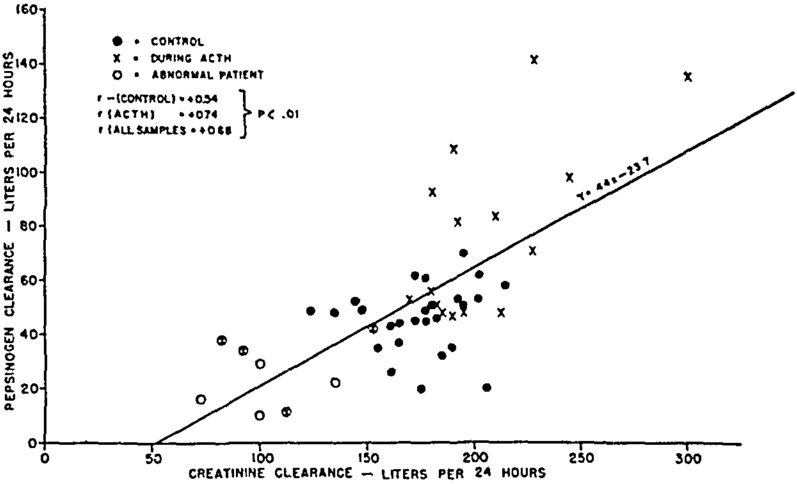

Fig. 3.—Relation between creatinine clearances (GFR) and pepsinogen clearances before and during ACTH administration.

being somewhat better during ACTH administration than in the control periods, but both these and the over-all correlation (r = + 0.68) are significant at the 1 per cent level.

DISCUSSION

The determination of the renal clearance of any substance requires accurate measurement of the *same* substance in the blood as in the urine. Moreover, with any extended period of urine collection, the clearance can only be valid if the blood level is determined at frequent intervals or alternatively a single blood level is adequate if there is no variation of any significance in the blood level during the period of urine collection

To comply with such requirements in measuring endogenous pepsinogen clearances the following facts appear to validate the method as employed in this investigation First, the proteolytic enzymes active at pH 1 5 to 2 5 in the blood and urine are measured by the same technique and are considered to be only pepsinogen[5] Second, the blood level of pepsinogen has been shown to remain constant throughout the day and from day to day, irrespective of food intake, dietary composition activity, or sleep,[11] with a coefficient of variation during the day of 8 per cent[11] In a group of 13 normal subjects studied by us, and reported elsewhere,[6] the coefficient of variation of the mean plasma pepsinogen concentrations in samples collected every 2 hours for 13 hours was 7 per cent In a similar group of 13 subjects receiving 25 units ACTH intravenously for 8 of the 13 hours, the coefficient of variation was 9 per cent

There are always objections to the use of endogenously produced substances such as pepsinogen and creatinine for clearance measurements, since their source of production may be influenced by the conditions imposed to alter their clearance However, since this study was designed to determine the causes for an increase in urinary pepsinogen occurring naturally, the endogenous substance had to be employed

The increased excretion of urinary pepsinogen found in the majority of patients with pyloric or duodenal ulcers[4] differs in one important respect from the increased output produced by ACTH or hydrocortisone,[6] viz, that in the former instance it results from an elevated plasma pepsinogen level, while in the latter the plasma levels are normal

In the usual ulcer subject increases in plasma and urinary pepsinogen probably reflect an increase in peptic cell mass[2] which appears to be a function of the duration of the disease[4] On the other hand, an increase in plasma pepsinogen may occur in ulcer subjects as a result of reversal of the pepsinogen secretion gradient, where a striking decline of pepsin secretion *into* the stomach is accompanied by a great increase of the amount of the proenzyme entering the circulation This phenomenon was observed in the healing stages and for some considerable time after healing of steroid induced peptic ulceration in 2 subjects reported elsewhere[1, 7]

Whatever the causes of an elevated plasma pepsinogen, the result is an increased pepsinogen load on the kidney which, with a fixed, normal clearance rate, results in an increased amount of pepsinogen excreted into the urine With ACTH and hydrocortisone, however, where the plasma pepsinogen levels remain normal, the opposite reason exists for an increase of pepsinogen in the urine, viz, an increased renal clearance of pepsinogen

The cause or causes of this increased clearance of pepsinogen with ACTH might be sought in one of two renal mechanisms probably responsible for the normal regulation of pepsinogen excretion. The difference between the average normal pepsinogen clearance of 44 L./24 hours and the glomerular filtration rate (GFR) of about 170 L./24 hours could be accounted for either by some glomerular impediment to the excretion of pepsinogen (molecular weight 42,000),[11] or by tubular reabsorption, or both, as is the case with plasma albumin (molecular weight 47,000).[12] The increases in GFR with ACTH reported here, of the same order of magnitude as those found by others[9, 10] using inulin clearances, and the fair correlation between pepsinogen and creatinine clearances suggest that changes in glomerular filtration play some part in the regulation of pepsinogen excretion. However, the differences between the relative increases in pepsinogen and creatinine clearances (48 and 13 per cent, respectively) suggest that increased glomerular filtration may not account for the whole increase in pepsinogen clearance with ACTH.

Support for tubular reabsorption as a mechanism in the control of urinary pepsinogen excretion may be derived from the increases in urinary pepsinogen caused by phlorhizin.[13] There is, however, no direct evidence at present available to indicate whether either or perhaps both the mechanisms suggested (an increase in the GFR or a decreased tubular reabsorption), is the means whereby ACTH increases the clearance of pepsinogen.

The fact that there is an increase in urinary pepsinogen excretion with ACTH and at the same time no significant fall in the concentration of plasma pepsinogen, implies that the amount of pepsinogen excreted in the urine is being replaced simultaneously into the plasma. This replacement could arise either from the leukocytes, in which there is a considerable amount of pepsinogen in man,[5] or, as seems more likely, from the stomach. In calculating the mean increase in urinary pepsinogen which could be expected during ACTH administration to result purely from the observed elevation of plasma pepsinogen (assuming a constant pepsinogen clearance), one arrives at a figure of only 11 per cent of the observed increase. Therefore, 89 per cent of the increase in urinary pepsinogen excretion resulted from the increase in renal clearance of pepsinogen. The converse calculation for the expected clearance estimated from the observed urinary pepsinogen excretion (assuming a constant plasma pepsinogen level) reveals a discrepancy of 11 per cent between observed and expected clearances during ACTH administration. Thus only 11 per cent of the observed increase in urinary pepsinogen could be ascribed to the elevations observed in plasma pepsinogen, which were small and inconsistent.

These observations suggest that the increase in urinary pepsinogen under the influence of ACTH is due principally to an increased renal clearance of pepsinogen and that the stomach maintains the blood level by some simultaneously acting mechanism which is independent of the secretory function of the stomach. This is confirmed by the lack of correlation between gastric pepsin secretion and the simultaneous measurements of plasma or urinary pepsinogen during ACTH administration.

SUMMARY

ACTH gel given for 5 to 6 days to each of 5 normal subjects caused an immediate and sustained increase of urinary pepsinogen excretion without substantially affecting gastric pepsin output or plasma pepsinogen concentrations There was, therefore, an increased clearance of pepsinogen by the kidney, a rise of 48 per cent which correlated with relatively smaller increases (13 per cent) in creatinine clearance (GFR) The increased pepsinogen clearance was due to both an increase in GFR and to probable changes in tubular reabsorption of filtered pepsinogen The absence of a fall in plasma pepsinogen concentration during ACTH administration implied that the amount of pepsinogen lost from the plasma by renal excretion was simultaneously being replaced from the gastric mucosa

The mechanism whereby ACTH increases urinary pepsinogen excretion differed from that responsible for the high urinary pepsinogen observed in the 6 patients with duodenal or pyloric ulceration In these patients the high urinary pepsinogen resulted from elevated plasma pepsinogen levels with a normal or even slightly reduced rate of renal clearance of pepsinogen

We wish to express our thanks to Mrs Esther Dullaert for her able technical as

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THE "MUCOPROTEIN FRACTION" (PHOSPHOTUNGSTIC ACID PRECIPITATE) IN TISSUE AND SERUM: A COMPARISON OF NORMAL FINDINGS AND FINDINGS IN LEUKEMIA AND LYMPHOMA

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QUANTITATIVE and, possibly, qualitative changes of one of the fractions of human serum, the phosphotungstic acid precipitate, are found in many diseases and particularly in hematologic disorders of neoplastic nature (lymphomas, leukemias).[1] This fraction has been shown to contain the largest amount of the serum mucoproteins as well as other poorly identified constituents and is generally defined as "mucoprotein fraction." Changes in content and composition of this fraction of serum have been shown to have diagnostic value, but their significance remains obscure. In particular, the available literature does not offer much information as to whether serum changes reflect similar abnormalities in mucoproteins of tissue. The only pertinent experimental observation to be found is by Catchpole,[2] who described parallel changes in glycoprotein content of serum and tissues in a group of mice with transplanted fibrosarcoma. We report in this paper an attempt to correlate content and composition of the phosphotungstic acid precipitate ("mucoprotein fraction") from serum and tissue (spleen) in patients with various hematologic disorders. A significant dissociation of values in serum and tissues was found in patients with lymphoma and leukemia.

MATERIALS AND METHODS

Collection of Specimens.—Serum and splenic tissue were obtained from 27 human beings suffering from a variety of diseases as shown in Table I. Serum was collected and stored as described previously.[1]

Both surgical and autopsy specimens of splenic tissue were studied. *Surgical specimens* were obtained from: (1) 5 patients with advanced lymphoma in whom surgery was performed because of intractable hemolytic anemia or thrombocytopenia with megakaryocytic bone marrow; (2) one patient with chronic malignant reticuloendotheliosis; (3) a miscellaneous group of 9 patients including 3 with acquired hemolytic anemia (idiopathic in one instance, due to fibrous congestion and to brucellosis in the remaining 2), 2 with idiopathic thrombocytopenic

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purpura (proved to be due to scleroderma and sarcoidosis, respectively, at histologic examination), one with aplastic anemia and enormously increased transfusion requirements, one with Cooley's anemia, one with congenital spherocytosis, one who suffered spontaneous rupture of the spleen, site of myeloid metaplasia. *Autopsy specimens* were obtained from 2 patients with lymphoma, 2 with acute leukemia, 5 with miscellaneous diseases (ruptured cerebral aneurysm, Laennec's cirrhosis of liver, brain tumor, chronic glomerulonephritis, and collagen syndrome).

TABLE I. SPLEENS STUDIED IN THE COURSE OF THIS WORK

TYPE	NO	SURGICAL SPECIMENS	AUTOPSY SPECIMENS
Normal	4	3	1
Lymphoma	7	5	2
Leukemia	3	1	2
Miscellaneous diseases	13	9	4
Total	27	18	9

Surgical specimens were perfused 5 times with cold saline immediately after collection In 2 instances specimens were washed rapidly with cold saline solution and frozen in liquid nitrogen for optimum preservation of all constituents.[3] Autopsy specimens were obtained within 2 to 4 hours after death. They were promptly washed free of blood with cold saline and immediately transferred to a Deepfreeze at -20° C, until used It was possible to separate lymphoma tissue from splenic tissue in 2 specimens and, thus, to study "mucoproteins" of the isolated lymphoma tissue. To check the reliability of our results, values obtained for surgical specimens were compared with those for autopsy material specimens in patients of the same disease group. Results were essentially similar. Also, several aliquots of the same spleen were collected with the different techniques mentioned above. Results of chemical analysis were well comparable. Thus, the technique of organ collection did not seem to influence the experimental findings

Preparation of Tissue for Chemical Analysis.—Frozen tissue was slowly thawed in an icebox at 4° C. for 2 to 6 hours. After thawing, a weighed aliquot of tissue was transferred to a wide test tube containing a volume of cold saline equivalent to 5 times the weight of tissue. Tissue was gently "broken" by squeezing it against the test tube wall by means of a large spatula. Centrifugation at 3,200 r.p.m at 4° C for 30 minutes followed; supernatant saline, red with hemoglobin pigment, was discarded. This process was repeated 5 times or until the supernatant fluid no longer contained visible pigment and tissue had assumed a yellowish tinge.* A volume of saline equal to the weight of tissue originally employed was then added. Tissue was mechanically ground in Van Potter's homogenizer for 20 minutes after adding 1 Gm. of sea sand per 10 Gm. of tissue. The ground material was transferred to glass tubes and centrifuged for 1 hour at 4° C, 3,200 r.p m. per minute. Supernatant yellow fluid, where no intact cells were found, was transferred by aspiration to other test tubes and used for all studies.

Chemical Analyses of Tissue—These included the determination of total protein (T.P), by a quantitative biuret technique[4]; the determination of total protein-bound hexoses (T Ph.), by the tryptophan method[5]; the determination of total hexosamine (T.Ha), with Rimington's method[6]; and the determination of total cholesterol (T Chol), with a modified Zlatkis technique. One half milliliter of homogenized tissue was added to 10 ml. of a boiling mixture of 1:1 acetone and alcohol. The mixture was filtered and total cholesterol was determined on the filtrate by the method of Zlatkis.[7] Total protein in the phosphotungstic acid precipitate (Mp.), was determined as described by Winzler et al.[8] As already mentioned, the phosphotungstic acid precipitate contains mucoproteins as well as other poorly identified constituents, although it is generally described as "mucoprotein fraction." Homogenized tissue suspended 1:1 in saline solution or serum was used. The following constituents were determined in the precipitated fraction: (1) hexoses (Mh.), by the tryptophan method described by Shetlar[8], (2) hexosamine (Mha.), by an application of Remington's method for serum[6]; (3) cholesterol (Mchol.), as described elsewhere.[9] Since tissue had originally been diluted to 50 per cent with saline all values obtained for tissue were multiplied 2 times to express absolute values.

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*Wright stained smears of the partially homogenized splenic tissue showed fairly well-preserved lymphocytes and lymphoblasts A mixture of lymphoblasts, reticulum cells, leukocytes, and giant cells was observed in the homogenized lymphoma tissue

Chemical Analyses of Serum.—These were carried out with the techniques previously described.[1, 3, 10]

Electrophoresis of Tissue Homogenates.—One tenth milliliter of tissue homogenate was transferred to a glass slide (1' × 3") and the slide so held that the fluid was uniformly distributed along its edge. The slide was then gently applied to a wet paper strip. This technique allowed fairly uniform distribution of the tissue homogenate on the paper strip. Migration was carried out by Durrum's method for serum proteins,[10] lipoproteins,[10] and glycoproteins.[11]

RESULTS

Normal Spleens (Table II).—A preliminary search of the available literature failed to find any data regarding concentration in normal splenic tissue of the chemical components considered in this article. Three surgical spleens and one autopsy spleen were then analyzed to obtain baseline values to interpret pathologic results. Tissue proteins appeared relatively rich in cholesterol and in total hexosamine as compared to serum.[1] Also the ratio of phosphotungstic acid precipitable protein to total protein was higher in normal splenic tissue than in normal serum.*

TABLE II. VARIOUS CONSTITUENTS OF NORMAL SPLENIC TISSUE

CASE NO.	DIAGNOSIS	T.P. (MG. %)	PROTEIN FRACTION			"MUCOPROTEIN FRACTION"*			
			T.PH (MG. %)	T.HA (MG. %)	T.CHOL (MG. %)	MP. (MG. %)	MH. (MG. %)	MHA (MG. %)	MCHOL (MG. %)
1	Normal	3,600	63	150	106	126	6.0	14.7	2.70
2	Normal	4,200	93	90	140	96	6.0	10.2	1.80
3	Normal	4,800	30	72	200	80	11.2	12.6	1.50
23	Cerebral hemorrhage	3,600	58	96	110	158	1.4	10.0	1.00
24	Chronic glomerulonephritis	2,000	38	90	130	94	8.4	10.5	1.65
25	Myocarditis	3,600	58	120	190	130	5.0	6.6	1.40
26	Cirrhosis of liver	3,300	45	120	130	108	9.3	9.3	2.25
	Average	3,585	55	105.4	113.7	113.1	7.47	10.55	1.76

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

Lymphoma Spleens (Table III).—Seven spleens were analyzed. It was possible in 2 instances to separate and analyze well-delineated, easily separable lymphoma nodules. Lymphoma spleen was richer in total protein than normal tissue. Total protein-bound hexoses and total hexosamine were also increased, in some quantitative relationship to the rise in total protein. Finally, the proteins of lymphoma tissue were rich in cholesterol. Phosphotungstic acid precipitable protein was also increased and relatively more so than total proteins. This fraction, however, appeared relatively poor in hexoses and hexosamine, while the amount of cholesterol showed slight relative increase.

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* idies may have helped in interpreting the high content of phosphoprotein in splenic tissue. Electrophoretic strips obtained from normal sence of albumin. Since albumin represents a large part of the total protein, its absence could help to explain the low total protein in splenic tissue as compared to serum. On the other hand, splenic tissue is rich in alpha-2 and especially beta-globulin *fractions which seem to contain or carry most of the protein moiety precipitable with phosphotungstic acid.* The latter fraction should then be increased in splenic tissue (Fig. 1).

TABLE III VARIOUS CONSTITUENTS OF SPLENIC AND ISOLATED TUMOR TISSUE IN LYMPHOMA

	PROTEIN FRACTION				MUCOPROTEIN FRACTION*			
CASE NO	TI (MG %)	TII (MG %)	TIIA (MG %)	TCHOL (MG %)	MI (MG %)	MII (MG %)	MIIA (MG %)	MCHOL (MG %)
4	6,000	100	176	380	270	9.20	15.60	2.54
5	6,270	72	147	280	300	11.40	9.90	3.30
7	8,400	102	207	300	257	13.05	9.30	3.03
8	4,800	112	144	330	210	10.20	10.80	2.50
9	4,200	64	102	260	242	7.70	6.20	1.98
10	5,000	70	118	200	260	8.50	6.60	2.44
Average	5,778	86.7	149	291.7	274	10.01	9.73	2.63
Isolated tissue								
6	3,500	80	160	147	240	6.40	5.00	1.42
7	3,600	70	122	145	254	8.40	6.00	1.50

For key to abbreviations see text.
*Phosphotungstic acid precipitate.

Isolated lymphoma tissue presented a somewhat different composition from lymphoma spleen. Total proteins were not increased, but were relatively

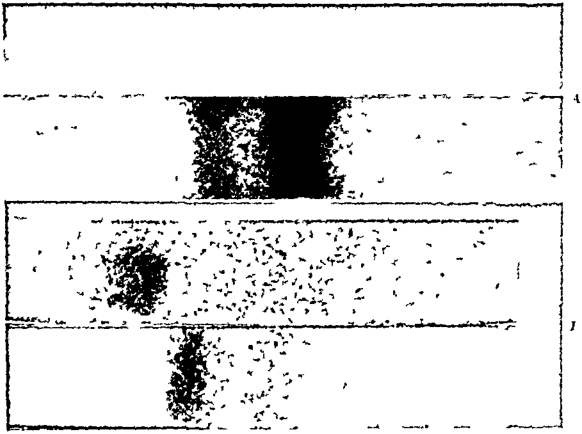

Fig. 1.—Electrophoretic pattern of soluble protein from normal splenic tissue and lymphoma tissue. *A*, Protein stain; *B*, glycoprotein stain.

In each group the upper strip represents normal splenic tissue, the lower strip lymphoma tissue. Note: (1) normal splenic tissue contains 2 peaks in γ and β position indicated by both protein and glycoprotein stain. No definite albumin component is present. (2) Splenic tissue from patients with lymphoma is much richer in stainable material. Major peaks are present in α_2, β_1, and β position; a small peak is found by protein stain in the α_1 albumin position.

rich in hexoses and hexosamine. Phosphotungstic acid precipitable protein, however, was greatly increased as compared to total protein; also, it was relatively poor in hexoses and hexosamine.

Electrophoretic studies of suspensions of homogenized lymphoma splenic tissue showed that, as with normal splenic tissue, there was preferential migration of proteins in the alpha 2 and beta zones. A new component, however, was found in position alpha-1, the significance of which is under investigation at present. Similar pattern was observed in separated Hodgkin's tissue (Fig. 1).

Leukemia Spleens (Table IV).—Results were very similar to those observed in lymphoma tissue. The only significant difference was a lower cholesterol content in the protein fraction.

TABLE IV. PROTEINS AND PHOSPHOTUNGSTIC ACID PRECIPITATE ("MUCOPROTEIN FRACTION") IN LEUKEMIA: COMPARISON OF BLOOD AND SPLENIC TISSUE FINDINGS

CASE NO	DIAGNOSIS		PROTEIN FRACTION T.P. (MG %)	T.PH. (MG %)	T.HA. (MG %)	T.CHOL. (MG %)	"MUCOPROTEIN FRACTION"* MP. (MG %)	MH. (MG %)	MHA. (MG.%)	MCHOL. (MG.%)
11	Histiocytic leukemia	Blood	7,200	147	140	200	310	42.50	12.75	1.30
		Spleen	7,050	138	234	146	120	6.8	13.60	1.20
12	Erythroblastic leukemia	Blood	7,000	160	140	160	123	20.91	18.70	2.00
		Spleen	7,800	126	156	150	306	11.10	14.73	1.80
13	Chronic malignant reticuloendotheliosis	Blood	7,700	145	130	180	130	34.30	14.50	3.80
		Spleen	5,550	60	216	170	295	11.40	11.85	2.25

For key to abbreviations see text.
*Phosphotungstic acid precipitate

TABLE V. PROTEINS AND PHOSPHOTUNGSTIC ACID PRECIPITATE ("MUCOPROTEIN FRACTION") IN SPLEENS OF PATIENTS WITH MISCELLANEOUS DISEASES

CASE NO	DIAGNOSIS	PROTEIN FRACTION T.P. (MG %)	T.PH. (MG %)	T.HA. (MG.%)	T.CHOL (MG %)	"MUCOPROTEIN FRACTION"* MP. (MG %)	MH. (MG %)	MHA. (MG %)	MCHOL. (MG %)
14	Acquired autoimmune hemolytic anemia	3,000	70	120	120	200	8.4	14.8	1.10
15	Fibrous congestive splenomegaly	8,250	63	186	206	270	12.45	22.8	3.75
16	Brucellosis; hemolytic anemia	6,900	117	195	185	339	13.35	22.8	3.45
17	Scleroderma thrombocytopenic purpura	8,700	90	249	207	345	10.5	12.3	3.30
18	Sarcoidosis, thrombocytopenic purpura	6,000	75	159	200	366	12.75	13.4	2.34
19	Aplastic anemia; hyperhemolysis	7,500	90	180	190	330	13.2	14.76	2.10
20	Congenital spherocytosis	4,830	90	135	210	300	6.4	9.8	3.48
21	Hemoglobinopathy	3,800	90	166	175	224	7.0	13.6	1.30
22	Myeloid metaplasia; rupture of spleen	6,150	111	180	220	357	7.6	11.2	3.03
	Average	6,125.5	88.4	174.4	190	303.4	10.18	15.05	2.54

For key to abbreviations see text
*Phosphotungstic acid precipitate

Spleens From Patients With Miscellaneous Diseases (Table V).—This group served as a control group to assess the specificity of the findings in lymphoma and leukemia. It included a number of conditions where splenectomy was performed for the relief of excessive hemolysis or of thrombocytopenia, or because of traumatic rupture of the organ. Independently of the pathology involved, splenic tissue was rich in total proteins and phosphotungstic acid precipitable fraction. Unlike lymphoma and leukemia, however, values of hexoses, hexosamine and cholesterol followed closely those of the two main fractions.

A Comparison of Values of Serum and Splenic Tissue in Lymphoma (Table VI).—A group of 4 patients were selected where splenic tissue and serum had been simultaneously obtained under optimal conditions (surgical specimens). Total proteins and hexosamine were slightly higher in serum than in tissue. Total protein bound hexoses were approximately twice as high in serum as compared to splenic tissue, the reverse was true of protein bound cholesterol. The phosphotungstic acid precipitable fraction was comparatively more abundant in splenic tissue than in serum, this tissue fraction, however, was poor in hexoses and hexosamine and very poor in cholesterol as compared to blood.

Table VI. Proteins and Phosphotungstic Acid Precipitate (“Mucoprotein Fraction”) in Lymphoma. Comparison of Serum and Splenic Tissue Findings*

		PROTEIN FRACTION				“MUCOPROTEIN FRACTION”†			
CASE NO		T P (MG %)	T H (MG %)	T HA (MG %)	T CHOL (MG %)	MP (MG %)	MH (MG %)	MHA (MG %)	MCHOL (MG %)
4	Blood	6,200	158	95	150	158	10.8	18	4.5
	Spleen	6,000	176	100	380	270	9.2	15.6	2.54
5	Blood	6,000	200	165	160	200	17.0	16	9.5
	Spleen	6,270	72	147	280	300	11.4	9.9	3.30
9	Blood	7,300	221	155	145	190	15.7	9.3	5.9
	Spleen	4,200	64	102	260	242	7.7	6.2	1.98
10	Blood	7,700	200	175	180	204	7.7	13.6	7.9
	Spleen	5,000	70	118	200	266	8.5	6.6	2.44
Normal	Serum	7,340	109.5	98.6	200	98.8	6.5	11.3	2.9
	Spleen	3,585	55	105.4	143.7	113.1	7.47	10.55	1.76
Lymphoma	Serum	6,800	197.2	147.5	159	188	12.8	14.22	6.9
	Spleen	5,367	95.5	114.2	292	269.5	9.2	9.52	2.56
Serum	Normal	7,340	109.5	98.6	200	98.8	6.5	11.3	2.9
	Lymphoma	6,800	197.2	147.5	159	188.0	12.8	14.22	6.9
Spleen	Normal	3,585	55.0	105.4	143.7	113.1	7.47	10.55	1.76
	Lymphoma	5,367	95.5	114.2	292	269.5	9.20	9.52	2.56

For key to abbreviations see text.

*Normal values are entered for purpose of comparison. The second part of the table shows comparative values of serum and splenic tissue in normal individuals and in patients with lymphoma. For splenic tissue, normal values are those of Table II; serum values are those recorded in other publications.[1,3,9]

†Phosphotungstic acid precipitate.

DISCUSSION

The interpretation of the findings presented in this article should be entertained with great caution. From a theoretical viewpoint, our conclusions were made difficult by the uncertainty of nomenclature and the chemical nature of mucoproteins. The phosphotungstic acid precipitate of tissue, even more than of serum, was likely to yield a fraction where the main constituents, mucoproteins, were mixed with a number of other tissue components. Moreover, techniques used involved repeated washing and manipulation of cells; thus, homogenates might have contained only more resistant cells; their number, in turn, may have changed greatly in number from normal to diseased state and in various conditions. Also, solubility and degree of recovery of proteins and other tissue constituents might have varied greatly because of noncontrollable influences. At their best, therefore, our results have mostly the value of comparing results in normal and in certain pathologic states.

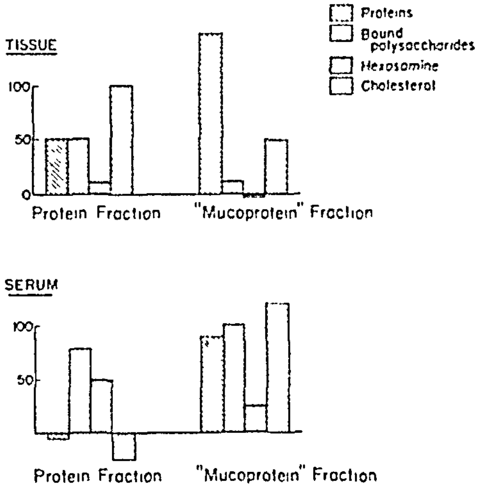

Fig. 2.—A comparison of various constituents of protein and phosphotungstic acid precipitate ("mucoprotein fraction") in serum and splenic tissue of patients with lymphoma.

Note: (1) Tissue fraction is rich in cholesterol, poor in hexosamine; serum protein fraction is rich in hexoses and hexosamine, poor in cholesterol; (2) "mucoprotein fraction" is abundant in both lymphoma serum and spleen; tissue "mucoprotein fraction" is poor in hexoses, hexosamine, cholesterol; serum "mucoprotein fraction" is poor in hexosamine, rich in cholesterol. Findings suggest in lymphoma: (1) abnormal distribution of various substances between serum and tissue; (2) possibly, abnormal composition of proteins and other tissue and serum constituents.

With these limitations in mind, a few comments are warranted. Total proteins, total nonglucosamine protein-bound hexoses and total cholesterol* were elevated in all diseases affecting the spleen, probably because of increased cellularity of the organ. Hexosamine, however, was elevated only slightly. Even more striking was the high content of phosphotungstic acid precipitable fraction in diseased splenic tissue. In lymphoma and leukemia, however, there were important additional qualitative changes, since this fraction was relatively poor in hexoses and hexosamine and, less strikingly, in cholesterol. Thus, quantitative and qualitative changes in the composition of the total protein fraction and of the "mucoprotein fraction" in pathologic splenic tissue as compared to normal tissue suggested significant changes in their composition, possibly specific for different disease states.

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*We have previously shown that accumulation of cholesterol in lymphoma tissue may be related to parallel increased content of cholesterol-carrying beta globulins.[3]

The comparison of tissue and serum values was also of some interest, although, perhaps, open to many objections. Our results showed a relatively fixed proportional distribution of total protein, phosphotungstic acid precipitate, and constituents of both fractions between tissue and serum from normal individuals or from patients with miscellaneous diseases In lymphoma, however, there was a striking dissociation of findings between splenic tissue and serum (Fig. 2). Total protein fraction of tissue was rich in cholesterol, *poor in hexosamine; total protein fraction of serum, on the other hand, was* rich in hexoses and hexosamine but poor in cholesterol. The phosphotungstic acid precipitate from serum was rich in hexoses and cholesterol. The tissue precipitate was poor in hexoses and cholesterol. The mechanism of the apparently altered equilibrium of certain serum and tissue constituents in lymphoma is being investigated at present.

SUMMARY

1. This article reports a study of some constituents of the total protein and phosphotungstic acid precipitable fraction ("mucoprotein fraction") of serum and splenic tissue in normal individuals and patients with lymphoma, leukemia, and miscellaneous disorders

2. In all pathologic cases studied splenic tissue was rich in total protein. The protein fraction of lymphoma tissue presented the strikingly distinctive feature of a very high cholesterol content. Total hexosamine was relatively low in splenic tissue of lymphoma and leukemia.

3 Pathologic splenic tissue was also rich in phosphotungstic acid precipitable material ("mucoprotein fraction"). When obtained from lymphoma and leukemic splenic tissue this fraction appeared relatively poor in hexoses, hexosamine, and cholesterol.

4 Comparison of splenic tissue and blood values showed a series of significant findings in lymphoma. Cholesterol of total proteins was high in tissue, low in blood. Also, total protein-bound hexoses were much higher in serum than in splenic tissue; phosphotungstic acid precipitate from tissue contained relatively less hexoses, hexosamine, and cholesterol than the corresponding fraction from serum.

5. Results suggested (a) quantitative and qualitative changes of total protein and "mucoprotein fraction" of serum and tissue in disease, (b) possibly, abnormal composition of these fractions in disease, particularly lymphoma.

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STUDIES ON PLATELETS XIX CAROTENOID PIGMENTS IN HUMAN PLATELETS

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IN THE course of unrelated experiments, it was observed by one of us that buttons of human platelets obtained from a large number of donors and stored at 4° or at -20° C would often assume a yellowish-brown color This observation suggested the presence of pigment precursors or of bound pigments in human platelets Further study indicated that an ethyl ether extract of these platelets[1] which had been stored for one month at -20° C contained yellow pigments and an ultraviolet absorbing material which was spectrally similar to vitamin A and in addition gave a positive Carr Price reaction Attempts to reidentify vitamin A in intact platelets or in their extracts has since failed Repetition of the work, however, has consistently revealed the presence of carotenoid pigments in extracts of platelets

MATERIALS AND METHODS

Preparation of Platelet Rich Plasma and of Suspensions of Isolated Platelets—Blood was collected from healthy or polycythemic individuals in plastic bags containing ACD solution † In several other experiments, blood was collected directly in silicone coated bottles containing 1/10 volume of 0 2 M sodium citrate Platelets and plasma from 10 different donors were used in these studies Platelet rich plasma and triply saline washed platelet buttons were obtained as described [2] Platelets were also obtained from rabbit blood collected in silicone coated syringes containing sodium citrate by direct arterial or heart puncture Techniques of preparation of rabbit platelets have been described earlier.[3]

Fractionation of Platelets and Pigment Extraction—The platelets from 1 pint of blood were suspended in 2 5 ml saline To this, 7 5 ml of ethyl ether containing 3 5 per cent ethanol was added The mixture was shaken vigorously for 5 minutes, and then centrifuged at 3,000 r p m for 30 minutes in cork stoppered test tubes Three layers were formed, as described earlier [1] After discarding the top layer, the remaining solution was stored at -20° C for 24 hours This material was thawed, centrifuged at 3,000 r p m for 30 minutes, and 3 new layers formed The top layer appeared yellow after this treatment, and was saved for further analysis An alternate technique consisted in adding 3 volumes of anhydrous sodium sulfate to a packed platelet button, and grinding until dry The resulting powder was extracted with petroleum ether, and then with acetone to remove protein bound pigments

Analysis of Pigments—The platelet extracts were transferred to petroleum ether, and chromatographed on alumina columns The pigments which separated were identified both

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by their adsorptive behavior and spectral characteristics. The concentration of the xanthophyll fraction (lutein) was obtained using the E (1%, 1 cm.)* value of 2,520 at 443 mμ. For the carotene fraction (a mixture of β carotene and lycopene), E (1%, 1 cm.) of 2,115 at 440 mμ, the crossing point of two compounds, was used.

RESULTS

Direct spectrophotometric readings of the petroleum ether extract of human platelets revealed the presence of two absorption peaks at 445 and 470 mμ (Fig. 1). Similarly shaped curves were obtained for both the acetone extract, and the ethyl ether extract, when these had been transferred into petroleum ether.

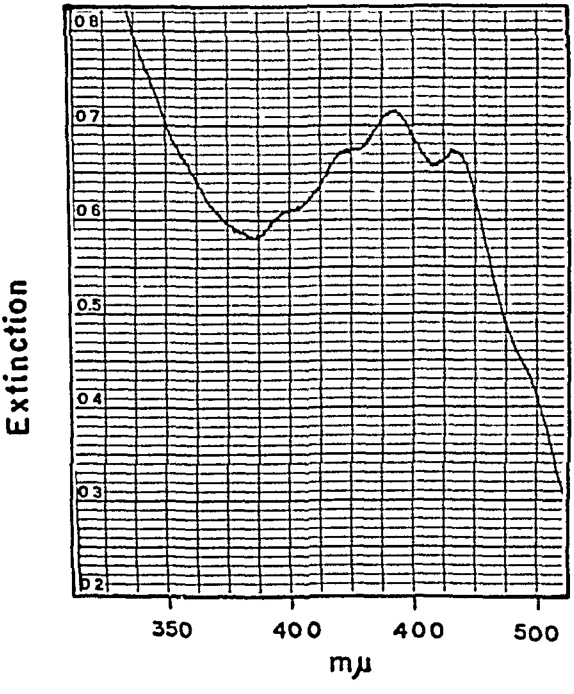

Fig. 1.—Spectrophotometric recording of a petroleum ether extract of human platelets. The extinction (ordinate) is plotted directly against wave length (abscissa).

Alumina chromatography at room temperature resolved these extracts into two colored fractions. The first, eluted with a solution of petroleum ether containing 4 per cent acetone, had an absorption spectrum indicative of a mixture of β-carotene and lycopene (Fig. 2, *a*).[4] When this fraction was saponified and then rechromatographed, it again was eluted with the 4 per cent acetone solution, indicating that there were no xanthophyll esters present. Following the elution of the first fraction, an additional yellow zone could be removed by the use of a petroleum ether solution containing 40 per cent acetone. This fraction, both by its adsorptive behavior and spectrum (Fig. 2, *b*), was identified as lutein.

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*The extinction (log I_0/I) of a solution containing 1 Gm. solute/100 ml. solution when read in a 1 cm. light path.

None of these extracts showed a positive Carr Price reaction, possibly because of the low concentration of the materials analyzed. Since, however, vitamin A had been found in a single previous preparation, the problem arose as to whether vitamin A could be formed from the carotenoids present in the platelets. Long term storage at –20° C., or incubation for 2 hours at temperatures up to 37° C. of either platelets or their extracts failed to be followed by the appearance of vitamin A.

Inasmuch as the plasma lipoproteins carry carotenoids,[1] it was felt that the carotenoids found in platelets might be due to lipoprotein contamination. Although this possibility cannot be eliminated rigorously, it appeared unlikely, since the amount of pigment recovered from the platelet extract was quite consistently proportional to the number of platelets used, irrespective of the presence of alimentary hyperlipemia. Moreover, in an additional experiment, platelets were obtained from the same donor before and after clarification of plasma from fats by the intravenous administration of 50 mg. of heparin. The amount of pigments finally obtained was very similar in both instances.

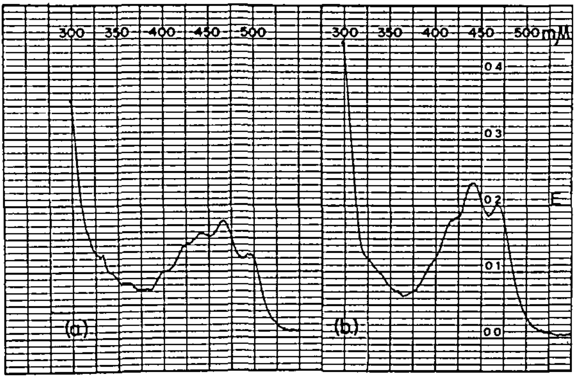

Fig. 2.—Spectrophotometric recording of a petroleum ether extract of human platelets after chromatographic separation on an alumina column. *a*, β carotene lycopene mixture eluted with 4 per cent acetone in petroleum ether. *b*, Protein eluted with 40 per cent acetone in petroleum ether.

Treatment of rabbit platelets as described indicated the presence of a pigment with λ max. at 398 and 420 mμ. The nature of this pigment is being investigated at present.

DISCUSSION

The findings presented in this article indicate that carotenoid pigments (xanthophyll and a β carotene lycopene mixture) are found in human platelets under certain experimental conditions. These pigments are among those also found in plasma. It was, therefore, important to compare the concentration of these carotenoids in platelets to the concentration in plasma. Platelets from one pint of blood contained 2.9 μg of xanthophyll and 2.5 μg of β-carotene-lycopene mixture. By comparable techniques[4] plasma from one pint of blood contained the same pigments in amounts of 94 and 170 μg, respectively. Since platelets usually represent 1 per cent by volume of plasma, a slightly higher concentration of these pigments in platelets than in plasma was apparent. Thus, platelets may concentrate carotenoids, contributing to their transport in the blood stream. This function of transport, thus far, has been considered limited to lipoproteins and chylomicrons.

Unfortunately, however, the relationship of carotenoid pigments to platelets could not be clarified further. It appeared unlikely that the presence of carotenoids in platelets might have been due to free diffusion from plasma, because the pigments exhibited relatively higher concentration in thrombocytes as compared to plasma. Also, multiple washings with water and even a single extraction with ether did not affect the concentration of pigments in platelets. It was possible, on the other hand, that platelets, whose structure could be visualized as a giant lipoprotein complex, might have adsorbed circulating carotenoid pigments. The resulting bondage was apparently a strong one, to be dissolved only by the complete destruction of the platelet body. A similar situation has been described for other constituents of platelets, like antifibrinolysin[5] and 5-hydroxytryptamine creatinine sulfate.[6]

Finally, the status of carotenoid pigments found in platelets was not clearly understood. Storage and strong chemical "treatment" were needed for their demonstration. Whether these procedures liberated or, perhaps, produced these pigments from other substrates; whether carotenoids were present in normal human platelets as such or, rather, as precursor substances remained undetermined.

SUMMARY

1. Human platelets have been found to contain lutein, lycopene and β-carotene. There is moderate concentration of the pigments in the platelet body as compared to plasma.

2. The finding is best explained by the known ability of platelets to absorb many constituents of the circulating plasma. Thus, platelets may contribute to the transport of carotenoid pigments in the blood stream.

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COAGULATION DEFECTS IN LEUKEMIA

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IT HAS been over thirty years since Minot and Buckman[1] published their classic article on bleeding in leukemia. Yet, in spite of a great deal of investigation, little has been learned of the etiology of the hemorrhagic diathesis in the various leukemias and other myeloproliferative disorders. Of course, in the presence of marked thrombocytopenia the cause is obvious and points the way toward the proper treatment. However, it is an uneasy situation for the clinician when his patient develops some hemorrhagic manifestation with a normal or even elevated platelet count. The etiology of the bleeding in this type of leukemic patient has been obscure and many explanations have been advanced. One of the earliest was decreased resistance of the vascular wall due to anoxemia and leukemic infiltration.[1] Although this may be a factor at times, it is not a common cause for the bleeding seen in the nonthrombopenic patient with leukemia.[2]

The possibility that the bleeding is due to qualitative platelet defects has been advanced without very firm evidence.[3] Why one patient bleeds while another, with comparable thrombocytopenia, does not is puzzling. Platelet and plasma serotonin determinations have not been of aid in this problem.[4, 5] Hypoprothrombinemia and AC globulin deficiency due to liver damage have been mentioned.[6] Again, these must be uncommon since most patients with chronic leukemia do not have abnormal liver function tests and have normal prothrombin times.[7] Fibrinogenopenia and fibrinolysins have been demonstrated in some cases,[8, 9] but this must be rare since Pisciotta and Schulz[10] failed to find one such instance in 40 patients with leukemia. Similarly, circulating anticoagulants are responsible in an occasional case only.[3] Finally, the presence of a serum polysaccharide has been postulated to account for the onset of bleeding in thrombocytopenic patients with leukemia.[11] However, fever and infection are not commonly associated with hemorrhagic manifestations in these patients.[3]

With all these theories, it is obvious that the cause for hemorrhage in leukemia is often obscure.[12, 13] As a result, the clinician is frequently at a loss as to appropriate therapy.

This study was undertaken in an attempt to elucidate the coagulation defect in leukemia and other myeloproliferative disorders.

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TABLE I

DISEASE	NO. OF PATIENTS	NO. OF PATIENTS WITH BLEEDING	AVERAGE PLATELET COUNT	AVERAGE BLEEDING TIME	AVERAGE CLOTTING TIME		NO. WITH ABNORMAL CLOT RETRACTION	AVERAGE PRO-THROMBIN TIME	AVERAGE PRO-THROMBIN CON-SUMPTION	NO. WITH AB-NORMAL TGT	PLATELET DEFECTS IN TGT	
					GLASS	SILICONE						
NORMAL	25		330,000	2'-3'	8'-15'	13' (6'-19')		13.5"	34.2"			
Leukemoid	3	0	421,000	2'40"		18'	1	16"	45.5"	0	Qual.	2
Polycythemia rubra vera	4	2	568,000	1'45"		23'30"	0	14.7"	31.5"	1	Qual.	2
Acute myelocytic leukemia	1	1	3,100	10'		20'	1	19.5"	12.5"	1	Quan.	1
Acute lymphocytic leukemia	1	0	45,000	3' 6"	13'	10'+	1	16.5"	18.5"	1	Qual.	1
Monocytic leukemia	2	1	115,000	4.5'		11'40"	2	14.5"	41.8"	2	Qual. Qual. and Quan.	1 1
Monomyelocytic (Naegeli) leukemia	1	1	3,000	10'–	40'+	40'–	0	17.5"	10"	1	Quan.	1
Chronic myelocytic leukemia	8	7	484,000	7'38"	10.1'	21'12"	5	14"	28.2"	6	Qual. Quan. Qual. and Quan.	3 1 2
Chronic lymphocytic leukemia	19	6	154,000	2'29"	7'	21'23"	6	13.7"	28.6"	12	Qual.	12

METHODS

Nineteen unselected patients with chronic lymphocytic leukemia, 8 with chronic myelocytic leukemia, one with acute lymphocytic, one with acute myelocytic, one with monomyelocytic (Naegeli) type, and 2 with monocytic leukemias were studied with a battery of clotting tests Four patients with polycythemia rubra vera and 4 with leukemoid reactions and myeloid metaplasia were also investigated

In addition, the same coagulation studies were performed on a group of 25 normal controls These included members of the staff and patients with no apparent hematologic disorder, liver disease, congestive failure, or other disease which might disturb the clotting mechanism One and frequently two normal controls were also done concurrently on days that patients with the myeloproliferative disorders were studied

Only siliconized needles and syringes were used Venipunctures were initiated with one syringe, which was then discarded, and only the blood obtained with a second syringe was used for the clotting studies

Platelet counts were performed by mixing equal quantities of venous blood (0 5 ml) and Rees Ecker solution[14] without dye Smears were then prepared as for ordinary blood smears and, after drying were stained with Wright's solution A modified Miller ocular disk reticulocyte technique[15] adapted for platelets was used in counting This method is excellent in that the results are reproducible with accuracy They may be rechecked at will since the smears are filed for further study

Prothrombin times were performed by use of human brain thromboplastin and resulted in a clotting time of 13 5 seconds normally Tests for accelerator globulin[3] and serum prothrombin conversion accelerator[3] were done when indicated

The prothrombin consumption test used was a modification of the Owren method[16] Two milliliters of blood were delivered into a standard tube which was inverted 10 times *and then placed in a 37° C water bath for one hour after clotting* The serum was then separated by centrifugation and a one stage prothrombin time done

Thromboplastin generation tests (TGT) were performed according to Biggs and Douglas,[17] with some modifications similar to those recently suggested[18] Incubation was *not carried beyond 6 minutes* The platelet suspension *as prepared for the thromboplastin* generation test in this study contained per cubic millimeter between 3 and 4 times the number of platelets in the patient's blood They were washed twice with saline and resuspended in a volume of saline equal to one third of the original platelet rich plasma suspension The platelet counts given in this paper were those found in whole blood The effect of the patient's platelets on thromboplastin generation was measured by using them in a system containing normal serum, barium sulfate treated normal plasma, and platelet poor plasma substrate as well as in a system containing only the patient's reagents The platelet suspension was also diluted or concentrated to correspond to an appropriate whole blood level and was used in both systems

The initial tests in every patient were performed by use of all of the patient's reagents both in his own substrate and in normal substrate plasma If there was no appreciable difference, the normal substrate was used for all subsequent tests in that particular patient Both substrate plasmas were used to aid in the detection of circulating anticoagulants of the antithromboplastin type In addition, tests for abnormal anticoagulant activity were performed, employing varying proportions of normal and patient's plasma[19]

Tubes containing 2 ml of blood were examined by visual inspection for clot retraction after one hour at 37° C and for fibrinolytic activity after 24 hours One milliliter aliquots of blood were delivered into glass and siliconized 1 × 10 cm test tubes for the whole blood clotting times by the Lee White method[13] These were done at room temperature

RESULTS

Table I summarizes the results obtained in the 39 patients studied It is to be noted that the average platelet count in the patients with chronic

TABLE II

DISEASE	PATIENT			AGE	HISTORY OF BLEEDING	PLATELET COUNT	BLEEDING TIME	CLOTTING TIME		CLOT RETRACTION	PROTHROMBIN TIME	PROTHROMBIN CONSUMPTION	THROMBOPLASTIN GENERATION	PLATELET DEFECT IN TGT
								GLASS	SILICONE					
Chronic lymphocytic leukemia	B.	♂	W	44	–	107,000	1′45″		30′	Poor	15″	14″	Abnormal	Qual.
	Ba.	♂	W	90	–	155,000	1′47″		18′	Poor	14″	17″	Abnormal	Qual.
	B., L.	♂	W	62	–	172,000	4′13″		22′	Good	15″	21″	Normal	None
	Bo.	♂	W	54	–	74,000	1′30″		19′	Poor	15″	13.5″	Normal	None
	C.	♂	W	64	+	97,000	1′13″		40′+	Poor	10.5″	25″	Abnormal	Qual.
	D.	♂	W	61	–	164,000	2′		8′	Good	15″	34″	Normal	None
	Du.	♂	N	49	+	161,000	3′		21′22″		12.5″	13.5″	Abnormal	Qual.
	F.	♂	W	64	+	183,000	2′		26′	Poor	13.5″	43.5″	Abnormal	Qual.
	Go.	♂	W	94	–	79,000	2′		18′	Poor	15″	36″	Abnormal	Qual.
	G.	♂	N	61	–	163,000	1′30″	7′	16′	Good	16″	81″	Normal	None
	Ha.	♀	W	76	–	263,000			22′		13.5″	36″	Normal	None
	H.	♂	N	38	+	140,000	2′ 5″		25′		12″	13″	Abnormal	Qual.
	Hu.	♂	W	60	+	81,000	3′15″		24′		14.5″	23″	Abnormal	Qual.
	J.	♂	W	68	–	297,000	1′30″		22′17″	Good	11.5″	26″	Normal	None
	J., S.	♂	N	62	–	140,000	2′40″		22′56″		14.5″	26″	Abnormal	Qual.
	M.	♂	W	60	–	329,000	5′22″		14′		12.5″	28″	Normal	None
	P.	♂	W	60	–	63,000	3′30″				13.5′	22.5″	Abnormal	Qual.
	S.	♂	N	76	–	155,000				Good	14″	33.5″	Abnormal	Qual.
	W.	♂	W	51	+	104,000	3′		11′	Good	13″	37″	Abnormal	Qual.
Chronic myelocytic leukemia	C.	♂	W	78	+	12,000	6′	12′	30′+	Poor	13.5″	14″	Abnormal	Quan.
	J.	♀	W	70	+	1,365,000		9′	6′30″	Good	14.5″	19″	Abnormal	Qual.
	K.	♂	W	75	+	7,000	10′+	9′30″	24′	Poor	14″	12.5″	Abnormal	Qual.-Quan.
	M.	♂	W	52	+	11,000	10′30″		16′30″	Poor	16″	14″	Abnormal	Qual.-Quan.
	P.	♂	W	63	+	1,825,000	4′30″		27′	Good	15.5″	30″	Abnormal	Qual.
	Pi.	♂	W	34	+	30,000	12′		21′	Poor	12″	13.5″	Abnormal	Qual.
	S.	♂	W	72	–	538,000	2′30″		24′	Poor	14″	33″	Normal	None
	W.	♂	W	34	+	182,000					12.5″	89.5″	Normal	None
Polycythemia rubra vera	E.	♂	W	49	+	451,000	2′30″		32′	Good	17″	32.5″	Abnormal	Qual.
	G.	♂	W	67	+	1,197,000			32′	Good	14″	19″	Normal	Qual.
	H.	♂	W	50	–	221,000	1′		18′	Good	14.5″	41″	Normal	None
	W.	♂	W	69	–	322,000			12′	Good	13.3″	13″	Normal	None
Leukemoid	A.	♂	W	65	–	428,000	1′		20′	Good	17″	65″	Normal	Qual.
	M.	♂	W	65	–	611,000	5′		14′	Good	15″	20.5″	Normal	Qual.
	T.	♂	W	71	–	223,000	2′		20′	Good	15″	51.5″	Normal	None

lymphocytic leukemia was reduced whereas it was somewhat elevated in the small number of patients with chronic myelocytic leukemia. In spite of this, the average bleeding time seems prolonged in the latter. However, from Table II it is apparent that the prolonged bleeding times occurred in patients with thrombocytopenia. The prothrombin consumption time was short in both groups. Platelet defects, as measured in the TGT in chronic lymphocytic leukemia, when present, were qualitative in nature. Concentration of the platelets in those patients with quantitative reductions to what would correspond to normal whole blood levels did not result in normal thromboplastin generation.

Table II lists the individual patients studied and the results of their clotting tests. Fibrinolytic activity was not found in any case. A circulating anticoagulant was thought to be present in only one individual. The latter was a patient (M.) with chronic myelocytic leukemia with an associated platelet defect. Thromboplastin generation was poor both in the patient's plasma and in normal substrate plasma. Correction was not obtained with substitution of any normal or combination of normal reagents. However, the test for anticoagulant activity was equivocal. One patient (J.) with chronic myelocytic leukemia had slightly deficient thromboplastin generation which was corrected only when either normal serum or plasma was used along with normal platelets.

In general, prothrombin times were not markedly prolonged. There were no instances of AC globulin or serum prothrombin conversion accelerator deficiency.

Of the 39 patients investigated 18 had a history of bleeding and, of these, 17 had at least one abnormal clotting test. The latter also included 16 with abnormal thromboplastin generation. Among the 21 patients with no history of bleeding 8 had abnormal TGT. On the other hand, one third of the patients with *poor thromboplastin generation tests gave no history of bleeding.*

TABLE III

DISEASE	ABN TGT + BLEEDING	NORMAL TGT + NO BLEEDING	NORMAL TGT + BLEEDING	ABN TGT + NO BLEEDING
Leukemoid		3		
Polycythemia rubra vera	1	2	1	
Acute myelocytic leukemia	1			
Acute lymphocytic leukemia				1
Monocytic leukemia	1			1
Monomyelocytic (Naegeli) leukemia	1			
Chronic myelocytic leukemia	6	1	1	
Chronic lymphocytic leukemia	6	7		6
Total	16	13	2	8

These results are listed in Table III. It is important to note that in the 24 patients with poor thromboplastin generation, 11 had normal prothrombin consumption. It is apparent that a normal prothrombin consumption time, especially in the presence of a prolonged prothrombin time, may be misleading and does not exclude an abnormality in thromboplastin generation.

Of the three patients with leukemoid blood pictures and myeloid metaplasia, none had a history of bleeding, although all had some abnormality in their clotting studies. One additional patient, not listed in the tables, had so-called "burned out" polycythemia rubra vera and a severe hemorrhagic diatheses. Thromboplastin generation was extremely poor. It was not possible, in this particular patient, to exclude the presence of myelocytic leukemia.

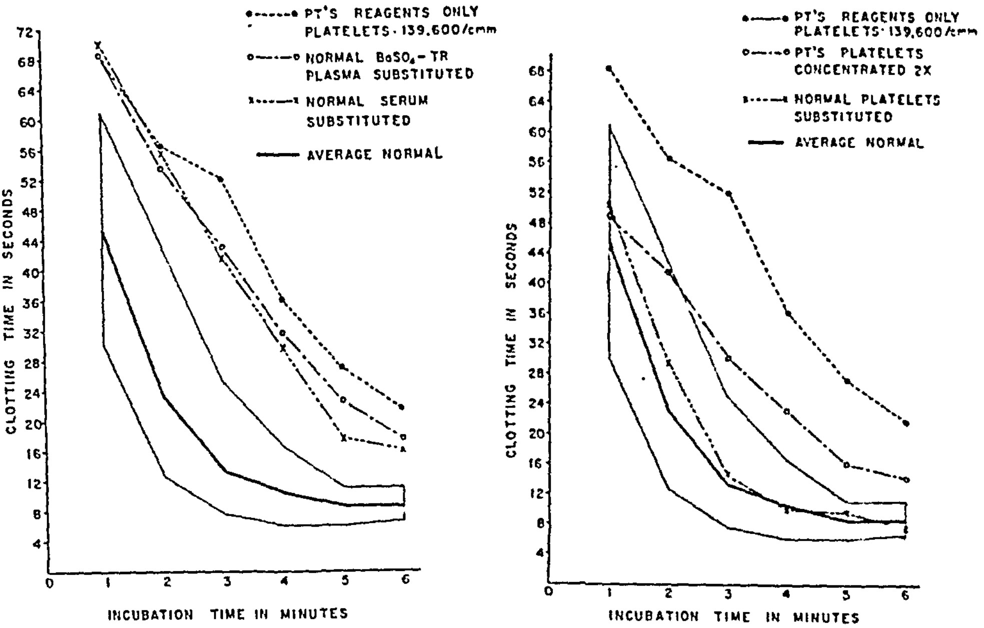

Fig. 1.—Patient (H.) with chronic lymphocytic leukemia and a qualitative platelet defect. Normal barium sulfate treated plasma and normal serum did not result in normal thromboplastin generation. Doubling of the patient's platelets improved the test only moderately. However, substitution of normal platelets resulted in normal thromboplastin generation. The shaded area indicates the range of thromboplastin generation in 25 normal controls.

As was to be expected, some of the platelet defects were found to be solely quantitative. However, qualitative defects as determined in the thromboplastin generation test were extremely common in these patients, particularly those with chronic lymphocytic leukemia. In this latter group, in fact, a quantitative reduction of platelets alone was in no case found to be responsible for an abnormal thromboplastin generation test.

Fig. 1 is an example of the type of platelet defect seen in chronic lymphocytic leukemia. Although the platelets were reduced in number, they were also qualitatively defective. Concentration to what would correspond to a normal value in whole blood did not result in a normal curve. Fig. 2 illustrates a similar defect in a patient with polycythemia rubra vera who had a slightly elevated platelet count.

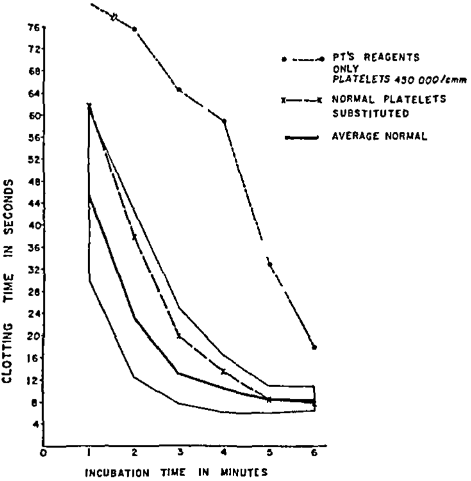

Fig 2—Polycythemia rubra vera Poor thromboplastin generation in a patient (L.) with a history of bleeding and a slightly elevated platelet count (normal 200 000 to 350 000) Normal thromboplastin generation was obtained when normal platelets were substituted

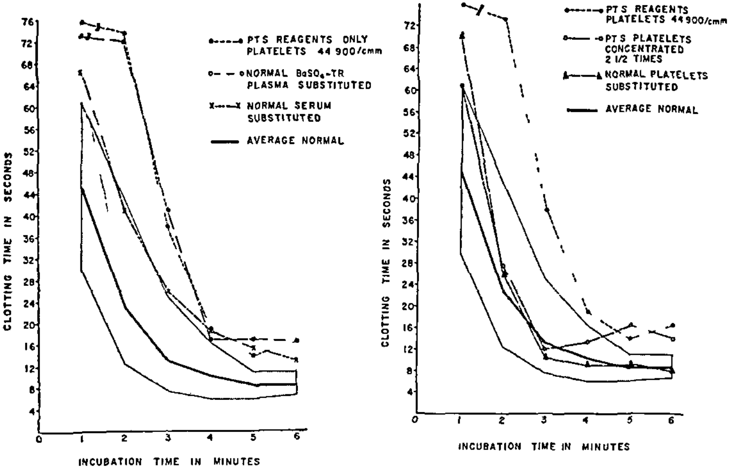

Fig 3—Acute lymphocytic leukemia Improvement in thromboplastin generation as the patient's own platelets were concentrated to what would correspond to a platelet count in the blood of 125 000 per cubic millimeter The platelet defect appears to be essentially a quantitative one

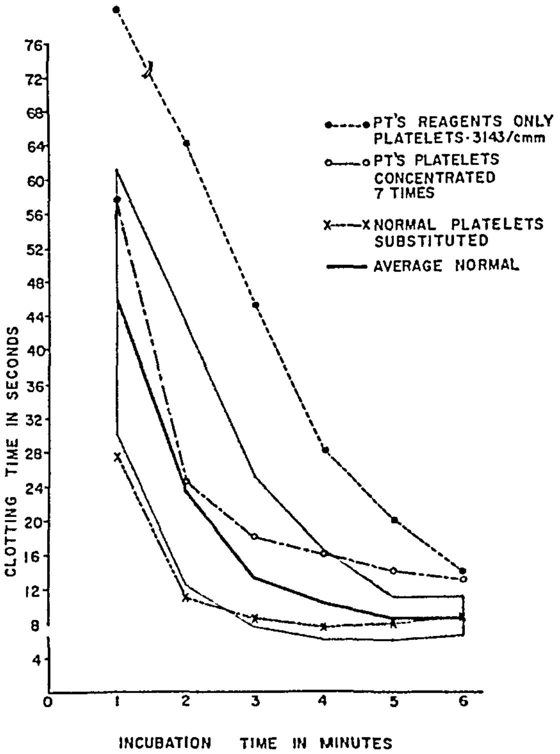

Fig. 4.

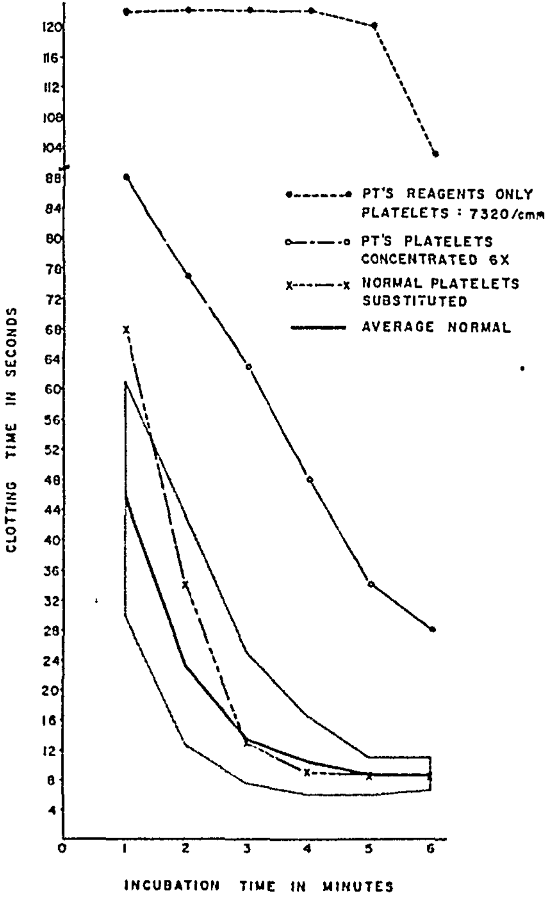

Fig. 5.

Fig. 4.—Acute myelocytic leukemia (Patient B.). Thromboplastin generation in the presence of marked thrombocytopenia. Although the patient's own platelets were concentrated only to a level of 22,000 per cubic millimeter, thromboplastin generation was almost completely normal.

Fig. 5.—Abnormal thromboplastin generation in a patient (K.) with chronic myelocytic leukemia. Marked improvement occurred after concentration of the patient's platelets to a level of approximately 11,000 per cubic millimeter.

Fig. 3 illustrates the effect on thromboplastin generation of a quantitative platelet defect in a patient with acute lymphocytic leukemia. Concentration of the patient's platelets in the system yielded almost a normal curve. A similar situation in acute myelocytic leukemia is shown in Fig. 4. Although the platelet count after concentration corresponded to a whole blood count of approximately 22,000 per cubic millimeter, thromboplastin generation was nearly normal. Fig. 5 is an example of the same type of defect in chronic myelocytic leukemia.

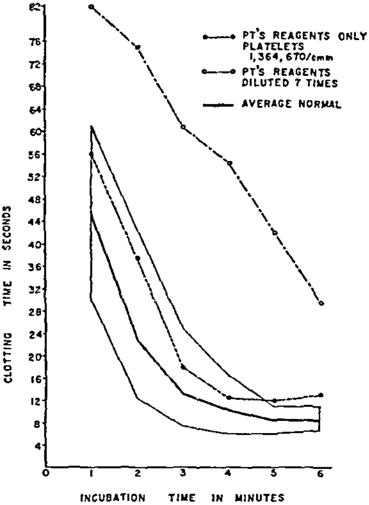

Fig 6.—Chror [illegible] *(Patient J). Demonstration of qualitative platelet defect only after tl*[illegible]*uted to a level of approximately 200,000 per cubic millimeter* [illegible] *as normal in the presence of the excessive numbers of patelets* [illegible]

There were three patients with platelet counts above one million per cubic millimeter. One patient with chronic myelocytic leukemia had a qualitative platelet defect as demonstrated in Fig. 6. The excessive numbers of platelets permitted good thromboplastin generation. However, with dilution of these platelets to a normal level, thromboplastin generation became inadequate.

Another patient with excessive platelets had an abnormal TGT (Fig. 7) even without dilution. Fig. 8 is an example of a patient with chronic myelocytic leukemia and elevated platelets in which, even with dilution, thromboplastin generation was normal. Just the opposite situation is shown in Fig. 9 in a patient with myelofibrosis where dilution resulted in poor thromboplastin generation.

DISCUSSION

The results of this study are in agreement with previous impressions[1, 5, 6] that there is no constant relationship between the platelet count and the incidence of bleeding. A patient with a normal or elevated platelet count may bleed and a patient with thrombocytopenia may never have any hemorrhagic manifestations. Three patients with excessive platelets (over one million) had bleeding histories. On the other hand, one patient (P.) with chronic lymphocytic leukemia and a platelet count of 63,000 had never had any bleeding. Generally, however, a patient with thrombocytopenia is more likely to bleed.[20] Yet a patient with a normal or high platelet count often will demonstrate a hemorrhagic diathesis if these platelets are qualitatively defective. In fact, thrombocythemia is frequently associated with bleeding in polycythemia rubra vera, chronic granulocytic leukemia, and so-called hemorrhagic thrombocythemia.[5, 21, 22] Patients with elevated platelet counts may demonstrate

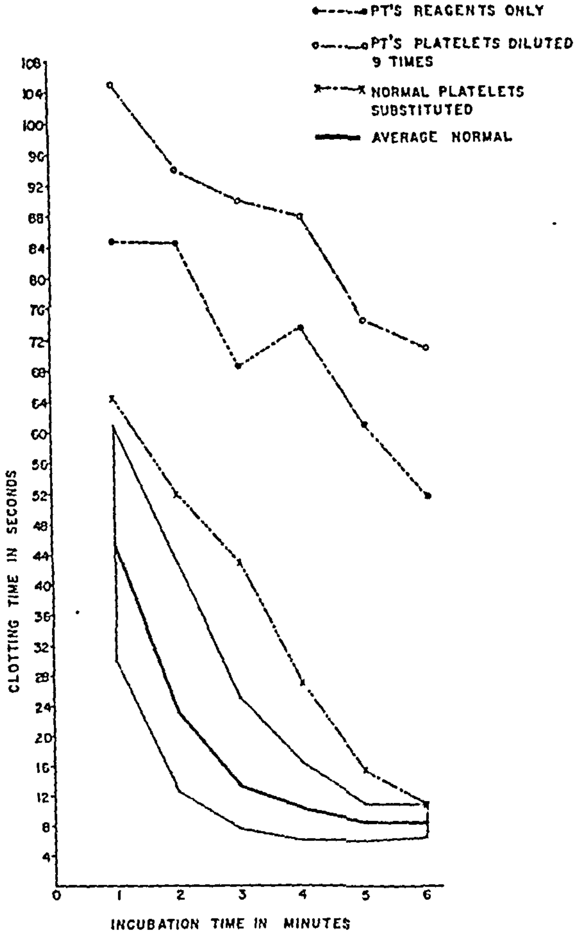

Fig. 7.—Abnormal thromboplastin generation in spite of a great increase in platelets (1,825,000 per cubic millimeter) in a patient (P.) with chronic myelocytic leukemia.

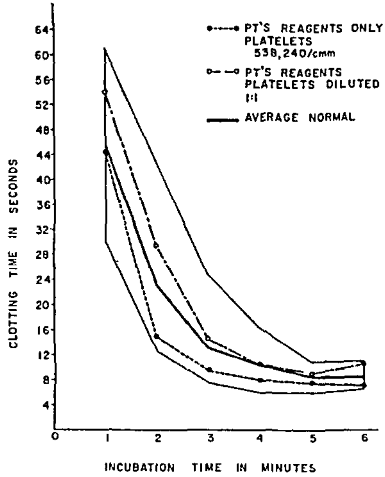

Fig 8 —Chronic myelocytic leukemia (Patient S) In spite of dilution of the platelets in this patient, thromboplastin generation did not become abnormal

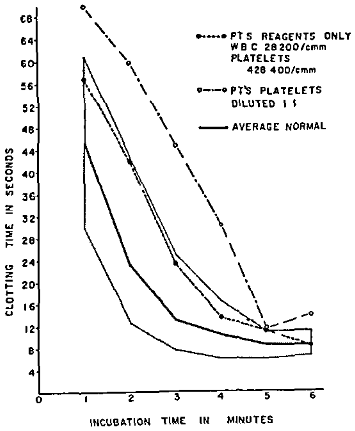

Fig 9 —An example in a patient (A) with myelofibrosis and a slightly elevated platelet count, where dilution of the platelets resulted in abnormal thromboplastin generation

abnormal thromboplastin generation only after dilution of the platelets to more nearly normal levels, as shown in Fig. 5. Hardisty and Wolff[5] have recently reported similar observations in patients with hemorrhagic thrombocythemia. Finally, excess normal platelets may even possess anticoagulant activity in vitro.[21]

From the results obtained, it is not possible to determine at what platelet level bleeding will occur. The value of 60,000 to 75,000, usually mentioned,[1, 20] is of little importance by itself. Low numbers of platelets may be functionally quite adequate, while, as mentioned above, excessive platelets may be associated with hemorrhage. Evidence for this may be seen in the results of the thromboplastin generation tests and observations on clot retraction. Similar variation of the critical platelet level was reported in 1946 by Aggeler and his co-workers.[20] In this connection, in regard to platelets, it is important to point out the lack of correlation among the results obtained in the thromboplastin generation test, clot retraction (by visual observation), and the platelet count. To gauge platelet function, all these tests must be performed in addition to the bleeding time and tourniquet tests.

Although deficient platelet function, particularly as measured in the thromboplastin generation test, often seems to be associated with hemorrhage, it is only presumptive etiologic evidence and may not be solely responsible for the bleeding. Other factors, not measured in this study or hitherto unrecognized, may be even more important.

Although not statistically significant because of the small numbers of patients studied, it is interesting to note that only qualitative platelet deficiencies were found in the group of patients with chronic lymphocytic leukemia. Sixteen of the 19 had low platelet counts. Abnormal thromboplastin generation tests in these were not corrected when their platelet suspensions were concentrated. In the other groups of patients both qualitative and quantitative defects were present. In the small number of patients with chronic myelocytic leukemia, variation in the levels of platelets from case to case was especially marked. The characteristic decrease in chronic lymphocytic leukemia and the variation in chronic myelocytic leukemia have been noted before.[1] The explanations for this and for the qualitative changes in the platelets are not obvious. However, functional deficiencies, including those involving the hemostatic activities of platelets, should not be totally unexpected. Biochemical and physiological abnormalities of the other blood cells in leukemia have been reported previously.[23, 24]

SUMMARY

1. A group of 39 patients with various myeloproliferative disorders, including chronic myelocytic and lymphocytic leukemia, were studied with a battery of clotting tests.

2. Of 18 patients with a history of bleeding, 17 had one or more abnormal clotting tests, including poor thromboplastin generation in 16. Four had excessive platelets and 14 were thrombocytopenic.

3 Among the 21 patients with no bleeding there were 10 with thrombocytopenia and 3 with increased numbers of platelets

4 Evidence was presented in agreement with previous impressions that there is no constant relationship between the platelet count and the occurrence of bleeding Within limits the functional hemostatic activity of platelets appears to be more important than merely the absolute number

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STUDIES ON SYNERGISM WITH TWELVE ANTIBIOTICS AGAINST THIRTY HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS

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MANY reports from hospitals in various parts of the world have indicated an increasing incidence of infections with antibiotic-resistant staphylococci. Therefore, combinations of 2 or more antibiotics are often used in treating staphylococcal infections to delay the emergence of resistant variants of staphylococci.

The antibacterial activity of antibiotics in pairs upon staphylococci has been studied to a limited extent. Rantz and Randall[1] reported the effect of 6 antibiotics in 15 combinations upon 33 strains of staphylococci in broth. They found that penicillin, streptomycin, and bacitracin were often "synergistic" when used together in pairs or added to chloramphenicol, oxytetracycline, or erythromycin. They defined synergism as killing within 24 hours with no secondary growth in subcultures, a phenomenon usually called the bactericidal effect.

Manten[2] found that streptomycin had a synergistic bactericidal effect with penicillin, chloramphenicol, tetracycline, or erythromycin against most of the 55 strains of staphylococci he tested. On the other hand, penicillin seemed antagonistic to any of the antibiotics except streptomycin. These effects were obtained by the use of antibiotic-impregnated paper disks on seeded agar plates and by observation of replica plates.

Kylin and Wall[3] studied the effect of penicillin and streptomycin upon the growth of 32 strains of staphylococci by the disk method. They found that these drugs often had a synergistic antibacterial effect. Waisbren[4] tested 8 antibiotics in pairs against 10 strains of staphylococci in broth with subcultures after 24 hours. He found no prevailing pattern of combined effects. Potentiation or interference with antibacterial activity between pairs of antibiotics was infrequent.

The purpose of the present investigation was to study the combined antibacterial activity of a larger number of antibiotics upon staphylococci isolated recently from patients in this hospital. The activity of the individual antibiotics was tested by the tube dilution method[5] with subcultures of each clear tube after 24 hours. The combined activity of 12 antibiotics in 66 pairs was tested against 30 strains of staphylococci by the paper strip-agar diffusion method of Elek[6] and Dye[7] with replica plating by the method of Lederberg.[8]

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MATERIALS AND METHODS

Source of Cultures—Thirty consecutive strains of hemolytic, coagulase positive *Staphylococcus aureus* were isolated from patients in this hospital in late 1954 Nine were from blood cultures, 9 from skin lesions 5 from urine, 3 from the nose or throat, 2 from body cavities, and 2 from wounds

Tube Dilution Method—Tube dilution antibiotic resistance tests were done on all 30 strains of staphylococci with 12 individual antibiotics * Doubling dilutions of the antibiotics in nutrient broth (Difco) ranged from 100 units or mcg to 0 39 unit or mcg per milliliter The bacterial inoculum was 0.5 ml of a 1 100 dilution of an 18 hour broth culture and was mixed with 0 5 ml of antibiotic in broth The bacteriostatic end point was recorded as the lowest concentration of antibiotic that prevented visible growth after 18 hours at 37° C All clear tubes were then subcultured by transferring one loopful with a 4 mm inoculating loop to a nutrient agar plate The lowest antibiotic concentration that prevented growth on subculture was recorded as the bactericidal end point All strains showing no growth in a concentration of 6 25 units or mcg per milliliter were arbitrarily called susceptible Those showing growth at 6.25 units or mcg per milliliter were recorded as resistant

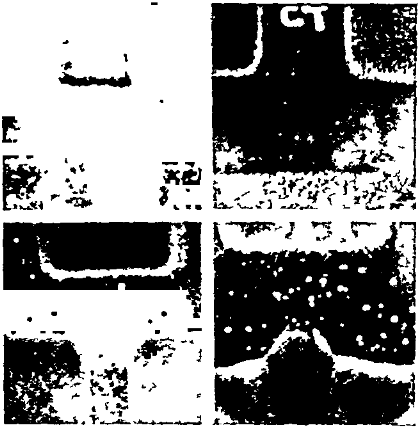

Fig 1 —Growth inhibition patterns with paired antibiotics for staphylococci by paper strip-agar diffusion method Upper left, no combined effect upper right, addition lower left synergism lower right, antagonism.

The Agar Diffusion Method—Nutrient broth and agar (Difco) were used throughout the study Imprint "stamps" for transplants to replica plates were prepared by fixing white velveteen to 1½ in pine disks 80 mm in diameter using polyvinyl acetate as the adhesive The e disks with tood cleaning and sterilizing at least one dozen times Five per cent phenol solution was used to sterilize the "stamps" immediately after use Standard 100 mm Petri dishes were used for all plate work

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*The antibiotics were dissolved in sterile distilled water They included the potassium salt of benzyl penicillin G (Chas Pfizer & Co) dihydrostreptomycin sulfate (Merck & Co
Inc.) chlortetracyclin oratories) oxytetracycline hydrochloride
(Chas Pfizer & Co) s. Pfizer & Co) chloramphenicol (Parke
Davis & Co) neomyc acitracin (The Upjohn Co) erythromycin
glucoheptonate (Eli L ly & Co) vancomycin (Eli Lilly & Co)
and novobiocin sodium of chloramphenicol and the tetracycline
group were prepared solutions were stored at 15° C for not
more than one week.

White blotting paper (substance 160) was cut into strips 6 mm. wide by 30 mm. or 75 mm. long. These strips were sterilized, dried, and placed in a fresh aqueous solution of antibiotic. The excess fluid was poured off and the strips were partially dried one hour at 37° C. They were used immediately.* The concentration of the antibiotics in the strips was adjusted for each bacterial strain to give a zone of growth inhibition 2 to 7 mm. wide around the strips.†

Plates containing 15 ml. of agar were inoculated with 0.5 ml. of a 1:100 dilution of an 18-hour broth culture and incubated at 37° C. for one hour (until the surface appeared dry). One paper strip (6 × 75 mm.) containing an antibiotic was then placed across the diameter of the plate. Three shorter strips containing other antibiotics were placed at right angles and touching the long strip. The plate was incubated 18 hours. The zones of growth inhibition were measured and the bacteriostatic growth pattern at the junction of the antibiotic strips was recorded photographically.‡ These were then interpreted as showing no combined effect (right angle), addition (rounded corner), synergism (corner cut off straight), or antagonism (tongue of growth) as shown in Fig. 1. The paper strips were then removed and an imprint of the growth on this original plate was made with a velveteen "stamp" and transferred to a sterile agar replica plate. This replica plate was incubated 18 hours at 37° C. The zones of growth inhibition were measured and the bactericidal growth patterns at the junction of the antibiotic strips were recorded.

RESULTS

The bacteriostatic susceptibility of the 30 strains to 12 antibiotics is given in Table I. There was a high incidence of strains resistant to the antibiotics commonly used prior to 1954 (penicillin, streptomycin, and the tetracyclines). Bacteriostasis was achieved at the lowest average concentration by novobiocin, vancomycin, and erythromycin. Table II represents the bactericidal activity of the same 12 antibiotics. The most active bactericidal antibiotics were vancomycin, neomycin, and bacitracin.

Table III reveals the action of 12 antibiotics in all the possible paired combinations by the agar diffusion method. The cases with no combined effect are not shown and represent the difference of the totals from 100 per cent. The antibiotics are arranged in order of decreasing addition of bacteriostatic activity. Combination of 2 antibiotics usually showed no potentiation if the staphylococci were resistant to one or both antibiotics. Thus the incidence of addition was low with streptomycin, penicillin, and the tetracycline group. Vancomycin, neomycin, and streptomycin showed greater additive potentiation of bactericidal activity than bacteriostatic activity. True synergism was infrequent with any drug combination and antagonism was rare. Bacteriostatic antibiotics did not antagonize the antibacterial action of the bactericidal antibiotics. Penicillin and streptomycin had no synergistic activity against these rather resistant strains of staphylococci.

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*Various methods of preparing antibiotic strips in advance were tried. The best method was to freeze them at -5° C. immediately after pouring off the excess fluid Attempts at drying at room temperature, in an incubator at 37° C. and by quick-freezing at -70° C. with drying under vacuum at room temperature all resulted in considerable loss of activity of some of the antibiotics.

†The concentration of the antibiotics per milliliter was as follows: penicillin 5, 10, 20, or 30 units; streptomycin 100 or 200 mcg.; the tetracycline group 100 or 200 mcg.; chloramphenicol 50 or 100 mcg.; neomycin 2 mg.; bacitracin 20 units; erythromycin 100 or 200 mcg.; cycloserine 200 mcg.; vancomycin and novobiocin 100 mcg.

‡The plate was exposed on Repro-Negative Kodagraph Extra Thin Paper, Eastman Kodak Co., Rochester, N. Y., with a photoflood lamp. The paper was then developed, dried, and mounted.

TABLE I. THE BACTERIOSTATIC ACTIVITY OF 12 ANTIBIOTICS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (TUBE METHOD)

	CONCENTRATION IN UNITS OF MCG. PER MILLILITER									
	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	>100
Novobiocin	28*	1		1						
Vancomycin	17	11	1	1						
Erythromycin	3	16	5	1	4					1
Chloramphenicol		8	14	7		1				
Bacitracin	1	2	4	23						
Neomycin	5	2	2	13	8					
Cycloserine				1	25	4				
Oxytetracycline	10				5	1	1	12		1
Chlortetracycline	10			1	3	2		5	9	
Tetracycline	9		1		1	2	6	5	3	3
Streptomycin	2	2	7	1					1	17
Penicillin	10								3	17

*Number of strains whose growth was suppressed. Median point enclosed by square.

TABLE II. THE BACTERICIDAL ACTIVITY OF 12 ANTIBIOTICS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (TUBE DILUTION METHOD WITH SUBCULTURE)

	CONCENTRATION IN UNITS OF MCG. PER MILLILITER									
	0.39	0.78	1.56	3.125	6.25	12.5	25	50	100	>100
Vancomycin	6*	3	4	9	1	2	2	1	2	
Neomycin	4	1	5	13	5	2				
Bacitracin	1		1	8	8	7	3	1		1
Erythromycin	2	1	1	3	1	10	5	2	1	4
Novobiocin	5				2	3	3	6	11	
Oxytetracycline	1			1	4	4	4	2	2	13
Tetracycline	1		1	1	3	4		2	3	15
Chlortetracycline	1			1				3	16	19
Streptomycin	2	2		1	1	2	1	2		19
Penicillin	4		1			3	2		1	19
Cycloserine					4	1		2	2	21
Chloramphenicol		2	1					1	1	25

*Number of strains killed. Median point enclosed by square.

TABLE III. ACTION OF 12 ANTIBIOTICS IN PAIRS ON 30 STRAINS OF STAPHYLOCOCCI (PLATE METHOD)

	PER CENT ADDITIVE		PER CENT SYNERGISTIC		PER CENT ANTAGONISTIC	
	BACTERIOSTATIC	BACTERICIDAL	BACTERIOSTATIC	BACTERICIDAL	BACTERIOSTATIC	BACTERICIDAL
Cycloserine	55.2	50.3	7.6	8.8	0.6	1.2
Chloramphenicol	53.9	53.0	2.7	4.5	0.6	1.8
Bacitracin	52.4	51.2	4.5	6.1	0.9	1.2
Vancomycin	51.2	68.2	7.0	5.8	0.6	0.9
Erythromycin	47.3	49.7	4.2	6.1	0.3	3.3
Novobiocin	43.6	41.2	5.8	5.5	1.8	7.3
Chlortetracycline	42.7	35.2	2.4	6.1	0.3	1.5
Penicillin	36.7	36.7	4.8	5.8	0.9	2.7
Neomycin	34.2	48.5	2.4	4.8	0.9	0.9
Tetracycline	33.3	36.1	4.2	6.7	1.5	3.3
Oxytetracycline	30.6	31.2	2.7	3.6	0.6	1.5
Streptomycin	22.1	32.1	2.4	5.5	0.6	0.9
Average	41.9	44.5	4.2	5.8	0.8	2.2

Table IV shows the incidence of addition of both the bacteriostatic and the bactericidal activities of 66 paired antibiotics against 30 strains of staphylococci.

TABLE IV. INCIDENCE OF ADDITION OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERICIDAL

		C	B	Cy	E	N	No	P	T	CT	S	OT	
		23	20	25	14	16	30	17	19	20	22	19	V
C	10		27	7	24	22	10	10	14	15	13	10	C
B	22	27		21	23	16	11	15	11	10	8	7	B
V	22	20	18		22	23	10	14	11	13	7	13	Cy
E	23	25	21	9		15	6	8	15	16	9	12	E
No	13	19	13	17	17		26	9	9	7	12	5	N
CT	15	15	15	23	15	11		14	7	5	11	6	No
P	15	12	16	10	12	12	10		10	8	9	7	P
N	25	18	14	9	6	13	8	9		8	6	9	T
T	12	15	10	14	15	9	9	9	4		4	10	CT
OT	15	10	9	16	11	8	10	8	3	9		5	S
S	10	7	8	11	2	12	5	8	4	4	2		
	Cy	C	B	V	E	No	CT	P	N	T	OT		

BACTERIOSTATIC

BACTERIOSTATIC

BACTERICIDAL

Key to symbols: Cy—cycloserine, C—chloramphenicol, B—bacitracin, V—vancomycin, E—erythromycin, No—novobiocin, CT—chlortetracycline, P—penicillin, N—neomycin, T—tetracycline, OT—oxytetracycline, S—streptomycin.

The numbers represent the number of strains of staphylococci that showed addition with a pair of antibiotics. Note that the total number in Tables IV, V, and VII is usually less than 30; the difference equals the number of cases with no effect.

TABLE V. INCIDENCE OF SYNERGISM OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF 12 ANTIBIOTICS IN PAIRS AGAINST 30 STRAINS OF STAPHYLOCOCCI

BACTERICIDAL

		T	E	B	CT	V	P	No	S	N	C	OT	
		3	6	1	2	2	1	5	3	1	3	2	Cy
V	2		3	3	0	4	1	1	3	3	1	0	T
No	6	0		0	1	0	3	0	2	2	1	2	E
P	2	3	7		4	3	4	4	1	0	2	2	B
B	1	3	4	0		3	2	0	4	4	0	0	CT
E	5	0	0	1	1		3	0	0	0	2	2	V
T	4	2	2	0	2	3		6	0	0	3	0	P
C	0	2	0	3	2	1	1		0	1	1	0	No
OT	1	5	0	0	1	2	0	0		2	1	2	S
CT	3	1	0	0	0	1	0	0	0		1	2	N
N	1	1	0	0	1	0	0	0	0	3		0	C
S	0	4	0	0	0	0	0	0	0	2	2		
	Cy	V	No	P	B	E	T	C	OT	CT	N		

BACTERIOSTATIC

BACTERIOSTATIC

BACTERICIDAL

See Table IV for key to symbols

Table V is a summary of the incidence of synergism of both the bacteriostatic and the bactericidal activities of all the possible combinations of the 12 antibiotics in pairs. The incidence of synergism was not as frequent as has been reported by others.[1-3] This may be the result of our more exact definition of synergism as well as the relatively high resistance of our hospital strains of staphylococci to many antibiotics.

Table VI gives a total of the data from Tables IV and V and shows a closer agreement with the high incidence of "synergistic" activity (potentiation) found by others.

Table VI. Incidence of Addition or Synergism of Bacteriostatic and Bactericidal Activities of 12 Antibiotics in Pairs Against 30 Strains of Staphylococci

BACTERICIDAL

		C	B	Cy	E	N	No	P	T	CT	S	OT	
		25	23	27	14	16	30	20	23	23	22	21	A
C	10		20	10	25	23	11	13	15	15	14	10	C
B	23	29		22	23	16	15	15	14	14	9	9	B
A	24	22	21		28	24	15	15	14	15	10	15	Cy
E	28	26	22	9		17	6	11	18	17	11	14	E
No	19	19	17	17	17		27	9	12	11	14	7	N
CT	18	15	15	24	16	11		20	8	5	11	6	No
P	17	15	16	13	13	19	10		11	10	9	7	P
N	26	18	15	10	6	13	11	9		8	9	9	T
T	16	16	12	16	18	11	9	9	4		8	10	CT
OT	16	10	10	21	13	8	10	8	3	9		7	S
S	10	7	8	15	2	12	7	8	6	4	2		
	Cy	C	B	A	E	No	CT	P	N	T	OT		

BACTERIOSTATIC

See Table IV for key to symbols.

Table VII. Incidence of Antagonism of Bacteriostatic and Bactericidal Activities of 12 Antibiotics in Pairs Against 30 Strains of Staphylococci

BACTERICIDAL

		E	T	P	C	OT	CT	Cy	B	A	N	S	
		6	5	1	4	2	2	0	1	0	0	3	No
T	1		1	3	0	0	0	0	1	0	0	0	E
B	1	1		1	1	0	0	2	1	0	0	0	T
P	1	0	0		0	2	0	0	0	1	1	0	P
N	1	1	1	0		0	0	0	0	0	1	0	C
Cy	0	1	0	0	0		0	1	0	0	0	0	OT
C	1	1	0	0	0	0		0	0	2	1	0	CT
A	0	0	0	1	0	0	0		1	0	0	0	Cy
OT	0	0	0	0	0	1	0	0		0	0	0	B
S	1	0	0	0	0	0	0	0	1		0	0	A
E	0	0	0	1	0	0	0	0	0	0		0	N
CT	0	0	0	0	0	0	0	1	0	0	0		
	No	T	B	P	N	Cy	C	A	OT	S	E		

BACTERIOSTATIC

See Table IV for key to symbols.

Table VII presents the incidence of antagonism of antibacterial activity, both bacteriostatic and bactericidal. The incidence of antagonism was generally very low. It was most frequent when the bactericidal activity of novobiocin combined with erythromycin, tetracycline, or chloramphenicol was measured.

DISCUSSION

As shown in Table I only 10 of the 30 strains of staphylococci were susceptible to penicillin. Cross resistance to streptomycin occurred with 80 per cent of the penicillin-resistant strains and 20 per cent of the penicillin-susceptible strains. Cross-resistance to chlortetracycline, oxytetracycline, and tetracycline occurred in 20 per cent of the penicillin-susceptible and 70 per cent of the penicillin-resistant strains. Cross-resistance between the 3 antibiotics in the tetracycline group occurred in all but one resistant strain.

The combined activity of any two antibiotics usually was not enhanced if the strain of staphylococci was highly resistant to one or both antibiotics. Therefore synergism was infrequent between penicillin, streptomycin, and the tetracyline group. As yet resistance to the other antibiotics is not frequent, chiefly because they have not been widely used. The ability of staphylococci to become resistant to the new antibiotics has been proved in this laboratory and others.[9, 10] The value of new antibiotics in combinations probably will diminish as resistant strains of staphylococci increase.

In the case of those antibiotics to which the 30 strains of staphylococci showed a wide range of resistance, the broth dilution and agar diffusion methods of determining resistance generally showed a fairly good correlation. When the range of resistance was small, the two methods often gave results that were not in close agreement.

The present study indicates that antibiotic combinations in general may be expected to have greater antibacterial activity against most hospital strains of staphylococci than one antibiotic alone. The potentiation is usually only additive and not truly synergistic. The use of combinations of antibiotics in treating staphylococcal infections seems to delay the emergence of resistant strains of the organism.[10] One need not fear that bacteriostatic antibiotics will interfere with bactericidal antibiotics.

The results shown in Table VI indicate that the most frequent enhancement of the antibacterial activity of antibiotics in pairs occurs with the newer antibiotics, to which the hospital strains of staphylococci are most susceptible. These combinations include novobiocin with vancomycin, bacitracin with chloramphenicol, and erythromycin with cycloserine.

Many of the reports by others using paired antibiotics against staphylococci are not in close agreement with the results of this study. The probable reasons for this disagreement can be found in the use of different methods, in the varying definitions of synergism and in the fact that our study was done with relatively resistant strains such as are now common in most hospitals.

SUMMARY AND CONCLUSIONS

1. Thirty hospital strains of *Staphylococcus aureus* were exposed to 12 antibiotics, both singly and in 66 paired combinations. Antibacterial activity was measured by tube dilution with subcultures as well as by agar diffusion from paper strips with replica plating. The combined effect of the paired antibiotics was judged from the growth patterns around the paper strips.

2. A high degree of resistance to penicillin, streptomycin, and the tetracycline group was frequent. Cross-resistance was common to these antibiotics Resistance was rare to the newer antibiotics.

3 Bactericidal activity was most marked with neomycin, vancomycin, and bacitracin

4 The 12 antibiotics in pairs rarely showed antagonism of bacteriostatic or bactericidal action True synergism was seldom found with the accurate paper strip–agar diffusion method Addition of both the bacteriostatic and the bactericidal activities was frequent with all strains of staphylococci susceptible to both antibiotics Antibiotics to which the strains of staphylococci were highly resistant had little or no effect in a combination

5 Enhancement of antibacterial activity against staphylococci was most frequent with the combinations of novobiocin with vancomycin, bacitracin with chloramphenicol, and erythromycin with cycloserine

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SERIAL SERUM GLUTAMIC OXALACETIC TRANSAMINASE (SGOT) AND ELECTROPHORESIS STUDIES IN THORAZINE-INDUCED JAUNDICE

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THE use of Thorazine (chlorpromazine hydrochloride, 10-[γ-dimethylamino-propyl] 2-chlorphenothiazine hydrochloride) in a great variety of clinical conditions has become widespread. Certain side effects have come to light, such as drowsiness, nasal congestion, constipation, rash, postural hypotension, and paralysis agitans. Of perhaps more serious nature is an alteration in liver function.

A number of cases of jaundice following the use of Thorazine have been reported.[1-4] Studies of liver biopsies[5, 6] have suggested that the anatomic lesion is of the obstructive type, with centrilobular distention of bile canaliculi. Menguy and his associates,[7] from experimental work in dogs, have suggested that the cholestasis is due to increased resistance of the choledochoduodenal sphincter, related to the depressing effects of chlorpromazine on duodenal motility. The occurrence of eosinophils in the cellular exudate usually found has suggested that the reaction to the drug may be on an allergic basis. The anatomic lesion is not unlike that described in the rare case of jaundice following the use of cinchophen,[8] thiouracil,[9] or methyltestosterone.[10]

The problem of distinguishing the jaundice occurring during Thorazine therapy from extrahepatic obstructive jaundice may be a difficult one. Liver function tests have indicated a raised direct and total bilirubin, and an increased alkaline phosphatase level. Recently, the determination of the amount of glutamic oxalacetic transaminase (GOT) in the blood serum has been proposed as a useful study in the differentiation of obstructive and cytotoxic jaundice.[11-13] Although in the jaundice of viral hepatitis or acute yellow atrophy exceedingly high values for SGOT are obtained, in obstructive jaundice the serum levels are increased only moderately. In a recent paper discussing SGOT changes in liver diseases,[14] it was stated in the conclusion that the serum levels were increased in Thorazine jaundice. Data were not reported.

RESULTS

We recently had the opportunity of following serially the chemical changes in the blood in 3 patients who became jaundiced in the course of therapy with Thorazine. We thought it might prove of value to report the findings in these cases.

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CASE 1.—A 34 year old white woman had received 25 mg. of Thorazine 3 times daily since March 5, 1956, for a depressive state. On March 25, she consulted her physician because of nausea, malaise, and fever. She was not clinically jaundiced. Physical examination was entirely negative. She was given tetracycline in doses of 250 mg. every 6 hours. Two days later, the patient returned, complaining of weakness and anorexia. Jaundice was then evident. Neither the liver nor the spleen were palpable. Thorazine was discontinued, she was placed on a high protein, high carbohydrate diet supplemented with large amounts of vitamins. At no time were the stools acholic. At the height of her hyperbilirubinemia, the urine contained bile, and test for urobilinogen was positive at a dilution of 1:160. Pruritus was minimal. By April 16, the patient had made a clinical recovery.

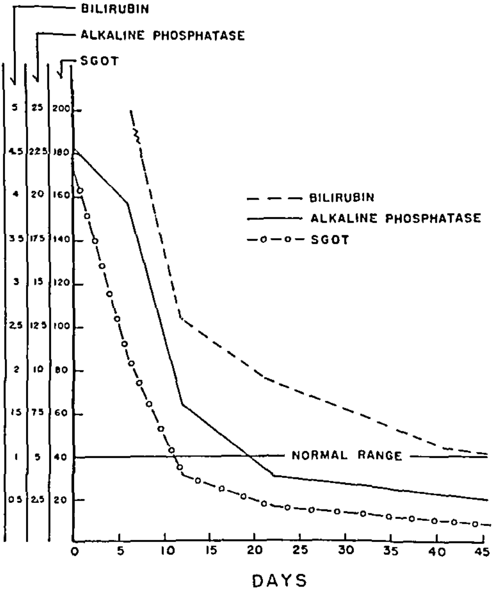

Fig. 1.—Case 1. Serial determination of bilirubin, alkaline phosphate, and SGOT.

The alterations in liver function are presented in Table I. The increases in bilirubin, serum alkaline phosphatase, and serum transaminase were maximal when determined 2 days after the clinical appearance of jaundice. The transaminase level was in the range that has been reported in obstructive jaundice, it returned to normal while the bilirubin and alkaline phosphatase were still significantly elevated (see Fig. 1). The thymol turbidity was never elevated, while the cephalin flocculation showed an insignificant rise at first, and then a slight increase when the bilirubin and enzyme levels had returned to normal. At all times the test for heterophil antibodies was negative. The chemical determination of blood proteins showed no significant abnormality. By paper electrophoresis, however, an inversion of the albumin globulin ratio was found, which was not reversed until the other liver function tests had returned to normal. The inversion was due largely to a first 10 days of the patient's jaundice. The bilirubin levels suggest that by the time this patient was admitted to the hospital, the jaundice was already decreasing. As examination of Table III and Fig. 3 indicates, the fall in bilirubin, alkaline phosphatase, and SGOT was more or less parallel, although the SGOT reached normal levels slightly before, and alkaline phosphatase slightly after, bilirubin. Cephalin flocculation, thymol turbidity, serum cholesterol, and heterophil antibody reaction were not significantly altered. There was slight elevation in Bromsulphalein dye (BSP) retention in a single determination at a time when SGOT, alkaline phosphatase, and bilirubin were still significantly elevated, though on the downgrade. Subsequently, the BSP returned to normal. Throughout the period of study, the gamma globulin was elevated.

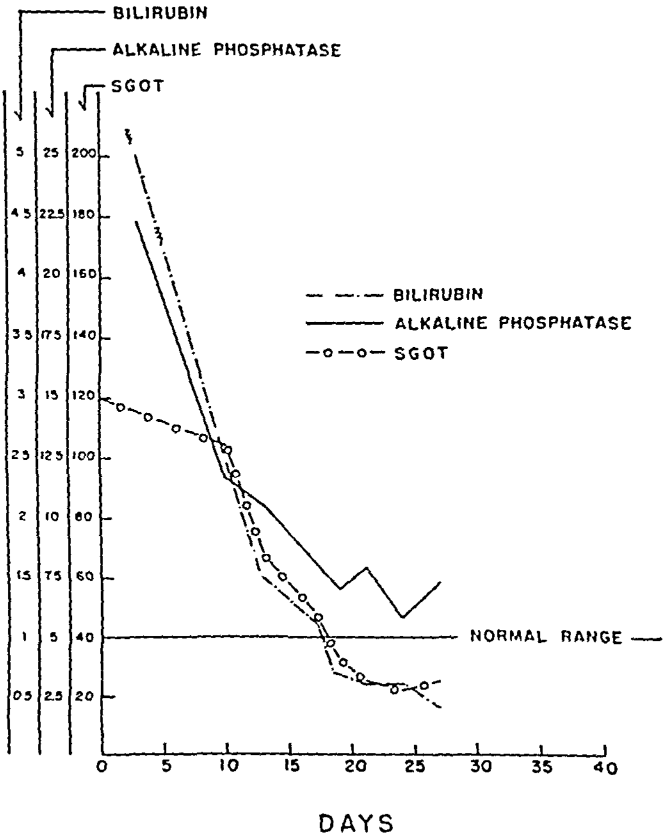

Fig. 3.—Case 3. Serial determination of bilirubin, alkaline phosphate, and SGOT.

On admission, a calculus obstructive jaundice was considered, but this became unlikely despite a nonfunctioning gall bladder on roentgenographic studies. One week after admission, she was transferred from the surgical to the medical service, where the differential diagnosis was considered to lie between infectious hepatitis of the cholangiolitic type and Thorazine hepatitis. The enlarged spleen was difficult to interpret. Studies of peripheral blood and bone marrow appeared to rule out a blood dyscrasia and hemolytic crisis. A liver needle biopsy, done on the twelfth hospital day, showed only pericentral cholestasis. No cellular infiltrate was found, and no necrosis. The histological picture gave no support to a diagnosis of hepatitis, of any origin, but, rather, was consistent with extrahepatic biliary obstruction. The patient developed an ascending urinary tract infection while in the hospital, for which she was treated. After almost one month in the hospital, she was discharged without definite diagnosis as to the nature of her jaundice.

DISCUSSION

In Cases 1 and 2, in which the diagnosis of Thorazine induced hepatitis appears fairly certain, examination of Tables I and II and Figs 1 and 2 shows the SGOT level moderately elevated shortly after the appearance of jaundice and falling off rapidly, with return to normal before the bilirubin or alkaline phosphatase values were again in the normal range By contrast, Case 3 (Table III and Fig 3) still showed an elevated SGOT some weeks after jaundice developed, a flatter type of curve, with more gradual return to normal, and closer correspondence with the curves for bilirubin and alkaline phosphatase levels It is unfortunate that it was not possible to have data during the period of early jaundice, so that the entire curve could have been plotted It would have been important to know the peak of SGOT Although the clinical diagnosis of Thorazine hepatitis was never discarded in this case, it does not appear a likely one Certainly, the liver biopsy lends no support The behavior of the SGOT differs in this case from the other two It would, doubtless, be desirable to have many more cases of Thorazine induced jaundice to establish a pattern If that demonstrated by the first 2 cases were consistently found, i e, sharp fall of a moderately elevated SGOT, it would prove an excellent and invaluable tool in establishing the diagnosis of Thorazine induced hepatitis It is hoped that this report will stimulate others to obtain such information

The addition of SGOT determination to the usual battery of liver function tests may prove of great value in helping to determine if jaundice developing in the course of Thorazine therapy is related to the drug, or to coincidental extrahepatic obstruction The rapid return of SGOT titer to normal appears to be similar to that described following acute myocardial infarction, and to that seen due to the toxic effect of drugs, e g carbon tetrachloride,[11, 12] and heavy metals upon the liver The dissociation of the temporal curves for SGOT, and alkaline phosphatase and bilirubin may prove of great value in the differential diagnosis

SUMMARY

Three cases of jaundice developing in the course of Thorazine therapy were followed by serial determinations of serum glutamic oxalacetic transaminase, blood protein fractions by paper electrophoresis, serum bilirubin, and alkaline phosphatase Of possible diagnostic help was the finding in cases of Thorazine induced jaundice of a moderate rise in SGOT level, and its rapid return to normal while the bilirubin and blood alkaline phosphatase levels were still significantly increased

We wish to express our appreciation to Drs J Schley, S Siegel, and E B LeWinn for the clinical data in their cases

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EXPERIENCES IN HUMAN BEINGS WITH AN IMPROVED FAT EMULSION FOR INTRAVENOUS ADMINISTRATION

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INTRODUCTION

THE need for a parenteral solution of high calorigenic value is well accepted The increased interest in geriatric problems as well as the extensive surgery that is possible today makes the perfection of such a preparation even more urgent However, the very fact that there is not available on the market today a fat emulsion for intravenous use emphasizes the problems in its production on a commercial basis These problems have centered about the physical stability of the emulsion and adverse reactions to it in the recipient

Numerous reports have appeared in the medical literature concerning both the preparation of various types of fat emulsions, as well as their use in animals and humans It is not my purpose to review this literature, however, the interested reader is referred to the excellent monograph by Freeman[1] on this subject The purpose of the present paper is to report our experiences with an improved fat emulsion prepared by The Upjohn Company† and contrast it to our previous experiences with a less satisfactory, but similar, product prepared by the same company Since recent large scale clinical reports have appeared with somewhat conflicting data on reaction rates utilizing different types of emulsions[2, 3] it is felt that this report will supply further information on which an appraisal of the practicability of such a product can be based

METHODS

The subjects infused with the fat emulsion were a heterogenous group of hospitalized patients on the wards of the Colorado General Hospital, Denver General Hospital and the Veterans Administration Hospital of Denver A small number of normal volunteers were studied on the metabolic ward of the United States Army Medical Nutrition Laboratory The majority of the hospitalized patients were on the Surgical Service and represent a great variety of surgical and medical problems They were infused sometimes before and sometimes after surgery No attempt was made to select patients, either according to disease or to severity of illness, except for 6 cirrhotic patients In many instances the patients were receiving other intravenous fluids, as well as various medications No attempt was made to alter the pre-existing medical regimen The fat emulsion

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From the U S Army Medical Nutrition Laboratory

The opinions expressed in this paper are those of the author and do not necessarily represent those of any governmental agency

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†The author wishes to thank Drs E A Hawk and R W Heinle of The Upjohn Co Kalamazoo Michigan for their generosity in furnishing the fat emulsion Lipomul

was given merely as an adjuvant. Many subjects had more than one infusion, although the majority received only one, since we were primarily interested in accumulating data on reaction rates. Only in special instances was the material given for therapeutic purposes over a long period of time.

The fat emulsion, Lipomul, contained 15 per cent cottonseed oil, 4 per cent glucose, 1.2 per cent soybean phosphatide, and 0.3 per cent Pluronic F 68. It was bottled in 600 ml. quantities which, according to the formula, provided approximately 900 calories per bottle. Since comparison will be made between an improved emulsion and an older one, reference will be made to the "old" emulsion and the "new" emulsion. *The formulation of the 2 emulsions is precisely the same.* The difference between the two resides in the purification of the soybean phosphatide incorporated into the new emulsion.

Although minor variations are unavoidable when studying such a hospital population, an attempt was made to standardize the actual administration of the emulsion as much as possible. The infusion was always started with a solution of 5 per cent dextrose dissolved in water. The emulsified fat was then admitted to the system via a needle in the rubber adapter. If other fluids were being given, the fat was preceded and followed for an hour by 5 per cent dextrose and water. Saline was carefully avoided in the system to prevent "breaking" of the emulsion which occurs in the (in vitro) presence of electrolytes. A total of 600 ml. of emulsion were given over a 3½-hour period, starting at a rate of 8 to 16 drops per minute for 20 minutes. If no reaction occurred, the rate was increased to 36 drops per minute for another 20 minutes. If the clinical condition at the end of this period of time permitted, the rate was increased to 68 drops per minute for the remainder of the infusion. Body temperature, pulse, blood pressure, and respirations were measured at 20-minute intervals for the hour before and the hour after the institution of the infusion. Thereafter, these vital determinations were performed at hourly intervals for the next 11 hours. A physician was in attendance throughout the entire infusion in each case.

When an adverse reaction occurred, the infusion was immediately stopped. If the situation demanded, appropriate medications were administered. In the great majority of instances, simply stopping the fat sufficed and reinstitution of the infusion was possible.

TABLE I. CHART DEPICTING REACTION RATES USING THE "OLD" AND "NEW" FAT EMULSION FOR INTRAVENOUS ADMINISTRATION ACCORDING TO NUMBER OF SUBJECTS AND NUMBER OF INFUSIONS

EMULSION	OLD (AUG., 1954–DEC., 1955)*				NEW (JAN., 1956–OCT., 1956)			
Number of Infusions	293				362			
Number of Subjects	110				151			
REACTIONS	NUMBER OF INFUSIONS	PER CENT PER INFUSION	NUMBER OF SUBJECTS	PER CENT PER SUBJECT	NUMBER OF INFUSIONS	PER CENT PER INFUSION	NUMBER OF SUBJECTS	PER CENT PER SUBJECT
Fever†	45	15	32	29	60	17	45	29
Chill	21	7	18	16	2	.6	2	1.3
Back and chest pain	38	13	33	30	0	0	0	0
Flushing	7	2	7	6	1	.3	1	.7
Nausea and vomiting	15	5	14	13	4	1	4	2.6
B.P. rise and fall	20	7	14	13	0	0	0	0
Dyspnea cyanosis	14	5	14	13	0	0	0	0
Shock	2	0.7	2	2	0	0	0	0
Rash	0	0	0	0	1	.3	1	.3
Miscellaneous (twitching, itching, hives, abdom. cramps)	6	2	6	5	3	.8	3	2
Over-all				51				33

*The author is indebted to Dr. Hugo Moeller and Dr. Morton Grossman for some of the data on the "old" emulsion used in this report.

†Over 100.6 orally or 101.6 rectally.

RESULTS

Table I depicts the recorded observations with the 2 emulsions in a comparable number of subjects. It is clear that there is a striking decrease in the over-all reaction rates with the new emulsion as compared to the old one. This is further exemplified by the fact that only 3 of 362 infusions could not be completed with the new emulsion, as compared with 23 of 293 that could not be completed with the old emulsion. Since those infusions which were discontinued were complicated by severe reactions, most of these patients were not reinfused; this makes the above comparison even more striking.

The various types of reactions encountered during the intravenous administration of emulsified fat have been described adequately by others. Often an attempt has been made to categorize some of the reactions into groups, e.g., "colloid reaction," and "histamine reaction." For purposes of clarity and comparison, each sign or symptom will be discussed separately, although it is recognized that many of the reactions may well be on a common etiologic basis.

TYPE AND INCIDENCE OF REACTIONS

It will be noted in Table I that data are given for the various reactions according to number of infusions and number of patients. This indicates that some subjects received more than one infusion. The consistently lower value for per cent per infusion as compared to per cent per subject reflects the often noted phenomenon of tolerance. Thus, most reactions occur on the first infusion and are much less likely to occur during successive ones.

Fever.—A rise in bodily temperature above the preinfusion morning temperature occurred in over 95 per cent of our subjects. However, since diurnal rise in body temperature is a common occurrence, and we were studying primarily a group of hospitalized patients who might be expected to have some fever from their primary illness, it was necessary that a significant febrile reaction be defined. A rise in temperature of 2° F. was not acceptable since many of our subjects had a morning temperature of 97° F. and a rise to 99° F. certainly could not be considered an adverse reaction. Hence, it was arbitrarily decided that, to be considered a significant febrile reaction, the temperature must rise in the 12 hour observation period at least 2° F. over the accepted normal rectal temperature of 99.6° F., or at least 2° F. over the control temperature if that control temperature was above 99.6° F. rectally.

Using the above criteria, 29 per cent of the subjects developed fever as a consequence of the infusion of the old emulsion as compared to 29 per cent with the new emulsion. The per cent per infusion is 15 and 17, respectively, for the two preparations. The fact that there is little difference in the febrile reaction rate between the two emulsions is considered important and will be referred to later. The nature of the febrile reaction, however, did differ somewhat in the two instances. With the new preparation, all of the significant temperature rises occurred as a late manifestation, often 2 to 4 hours after the infusion had been completed. Although this was often true with the old emulsion, 13 per cent of the febrile reactions did occur during the course of the infusion. However, the height of the temperature rise was not significantly different (Fig. 1).

It was interesting that, in most instances, the subjects were unaware of the fever except when it was accompanied by a chill or was markedly elevated.

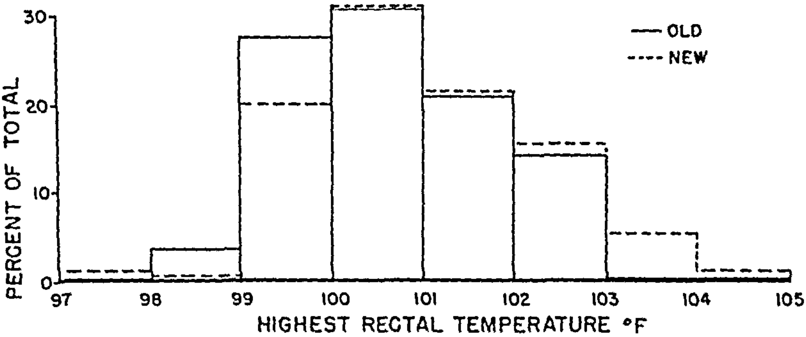

Fig. 1.—The incidence of the highest rectal temperature which occurred as a result of the first infusion of an "old" and "new" fat emulsion.

The duration of fever was variable but, for the most part, lasted only an hour or two. It was most common for the temperature to rise and fall rather rapidly.

Chill.—Sixteen per cent of the subjects with the old emulsion as compared to 1.3 per cent with the new one experienced chill as a complication of intravenous fat. The chill was delayed in the two subjects receiving the new emulsion.

Pain.—Back pain was the second most common reaction with the old material. It occurred in 30 per cent of the subjects and 13 per cent of the infusions. This type of reaction has not been observed in any of our patients receiving the new preparation. The reaction, when it occurred, was acute, often appearing after only a few drops of the material had been given and always within the first few minutes. Most subjects described it as a colicky or griping pain in the lumbosacral area. Rarely, it would occur across the chest or down the legs or arms. It was a frightening experience for the patient, but fortunately would subside within a few minutes after discontinuation of the infusion. No objective findings accompanied the pain and, as far as we know, no subsequent disability in any form occurred. Reinstitution of the infusion was possible in all instances without a recurrence of the symptom.

Flushing.—Six per cent of the subjects and 2 per cent of the total infusions were complicated by a flush with the old emulsion, while only one patient out of 151 experienced this phenomenon with the present one. Although uncomfortable to the subject, the flush disappeared promptly with temporary abandonment of the infusion.

Nausea and Vomiting.—This set of adverse reactions occurred in only 2.6 per cent of our subjects with the new preparation as contrasted to 13 per cent of the group receiving the other. Nausea and vomiting generally occurred after the infusion had been completed, although on several occasions one or the other was the cause of permanent discontinuation of the fat. The effect of the intravenous fat on appetite was difficult to assess. Some patients would eat heartily immediately after completion of the injection, while others complained of anorexia. In the latter case, the appetite had always returned by the evening mealtime.

Blood Pressure Variations.—Again, arbitrary limits had to be placed on those variations in blood pressure which would be considered abnormal and, therefore, a reaction to intravenous fat. A rise of 40 mm. Hg systolic or 20 mm. Hg diastolic or a fall in systolic blood pressure of from 20 to 40 mm. Hg, depending upon the control level, was considered a reaction. Utilizing these criteria, 13 per cent of the subjects and 7 per cent of the infusions were complicated by significant blood pressure changes when the old emulsion was administered. There were no such changes observed with the new emulsion. Of all of the various reactions formerly observed, the rise in blood pressure seemed to be most closely related to rate of infusion and almost always could be lowered by decreasing the flow.

Dyspnea and Cyanosis.—The symptom-sign complex of dyspnea and cyanosis occurred in 13 per cent of the subjects and 5 per cent of the infusions when the old emulsion was administered. None of the patients were thus affected with the new preparation. These reactions occurred early in the course of the infusion and often were severe enough to prompt permanent discontinuation. Appropriate medications such as antihistamines, adrenalin, aminophylline, and oxygen were utilized on occasion as necessary. Although it would be difficult to state definitely which medicament, if any, was effective on such a small group of patients, prompt discontinuation of the emulsion undoubtedly was most important.

Shock.—A full-blown picture of anaphylactoid shock was seen in 2 of our patients receiving the old emulsion. Both reactions occurred within the first 3 minutes of the infusion. Fortunately, both patients survived without any obvious sequelae. However, there is no doubt that, without immediate attention in both instances, a dire outcome could have been expected. No such reactions have been encountered with the emulsion presently employed.

Rash.—This reaction had never been observed until recently in one child receiving the new emulsion. It was of the erythema multiforme variety, moderately pruritic, appearing after approximately 30 minutes of the injection time had elapsed. Attempts were made on 5 different occasions to administer the fat, but the rash appeared each time. Prompt subsidence of the erythema would follow discontinuation of the emulsion. Prior and simultaneous administration of intravenous antihistamines was ineffective. Plans to skin test the child with each ingredient of the emulsion were interrupted by discharge from the hospital.

Miscellaneous.—Two bizarre reactions were observed with the new preparation which require further mention. A 34-year-old woman with advanced Laennec's cirrhosis of the liver was infused on 4 different occasions. With each infusion she complained of a nagging backache, which appeared toward the end or after the end of the infusion and lasted for several hours. The reaction was accompanied by gross tremor of the hands and head, some vomiting, and a moderate rise in temperature. When it occurred during the infusion, discontinuation of the fat had no effect. Past history revealed that the patient had made numerous visits to the receiving ward because of gross tremors of the body and had been admitted to the hospital on one occasion for hysterical paralysis of the legs. In this instance, an adverse reaction to the intravenous fat undoubtedly occurred; however, it was clouded by an overtone of hysteria. It is interesting to note that of the 11 patients with cirrhosis of the liver who were studied, this is the only reaction other than fever that occurred in the group.

The second patient was an 89-year-old man with severe generalized arteriosclerosis and arteriosclerotic heart disease. After 480 ml. of the infusion had been administered without mishap, 30 mg. of codeine sulfate were given for restlessness. Semistupor, accentuated by Cheyne-Stokes respiration, supervened within 30 minutes and the fat emulsion was discontinued. By late afternoon, the patient's condition had returned to that which was maintained prior to the infusion. In this instance the role of intravenous fat in the production of the semistupor is not clear.

In the total of 655 infusions there was no mortality and no permanent disability from any acute morbidity.

ATTEMPTS TO ALTER REACTION RATES

Interest in effecting a reduction in reaction rates was motivated not only from a therapeutic viewpoint but also in an effort to learn more about the mechanisms of the reactions. An insight into the latter might indicate directions to pursue in the production of a more suitable fat emulsion. Since most of the reactions occurred with the old emulsions, attempts to alter the reaction rates were performed in patients receiving that preparation.

Rate of Infusion.—Previous work in this laboratory in normal human volunteers showed that varying the time required for the infusion from an average of 131 minutes to 356 minutes did not affect the febrile response as measured by fever indices.[4] In the present study, an analysis of the correlation between the initial rate of infusion and the occurrence of back pain in 81 subjects revealed a negative value ($P > 0.50$). However, flow did seem to be correlated with elevation of blood pressure and since so many of the severe reactions, such as shock, dyspnea, or cyanosis, occurred early in the infusion period, we concur with others that all infusions of emulsified fat should be started slowly and the subject should be closely observed at the beginning of the injection.

Repeated Infusions.—It has been commonly observed that repeated infusions in the same person result in less frequent adverse reactions. This tolerance or tachyphylaxis was evident in our experience, although by no means invariably so. That tolerance to the febrile reaction develops is evident in Fig 2. However, one of the patients had an elevation of oral temperature above 102° F on each of 6 successive infusions. Another subject had an elevation of rectal temperature to 101.6° F only on the fifth day of successive infusions. The tolerance was more consistently observed in the case of back pain, flushing, nausea and vomiting.

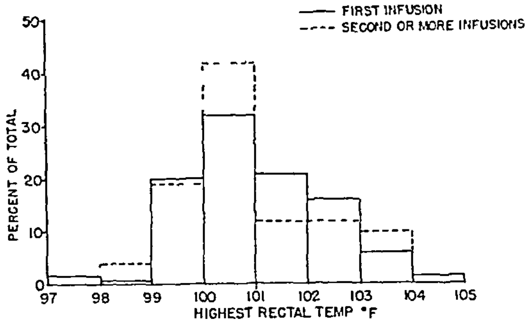

Fig 2.—A distribution graph depicting the incidence of the highest temperature recorded during a 12 hour period following the intravenous infusion of 600 ml Lipomul on the first occasion and succeeding ones.

Use of Antihistaminics.—Many of the immediate reactions resemble in some respects a histamine like response. With this in mind, 14 patients were studied with infusions of intravenous fat one week apart. All patients received 50 mg of Benadryl intravenously 15 minutes before and again intravenously halfway through one of two infusions. Nine of the 14 received Benadryl with their second infusion. In this small series of patients, wherein each subject serves as his own control, the incidence of reactions of all types was 35 per cent with Benadryl and 50 per cent without. The slight decrease in reaction rate was not considered significant enough to pursue further.

Addition of Serum Albumin.—Seventeen patients received an infusion of the emulsified fat to which had been added 18 ml of a 25 per cent human serum albumin solution. Nine of these subjects were also infused a second time, one week apart, with an unsupplemented bottle of emulsion. Thirty per cent of the 17 patients experienced some type of reaction with the serum albumin fat mixture, as compared to the over all reaction rate of 51 per cent in the 110 subjects receiving the old emulsion. Of the 9 subjects who had 2 infusions, 3 developed reactions with the serum albumin fat mixture and 4 had reactions with the regular material.

DISCUSSION

The remarkable reduction in adverse reactions with the exception of fever which occurred coincident with the purification of the soybean phosphatide used

4. The instability of the emulsion to extreme environmental conditions, electrolyte solutions, and amino acid solutions would seem to point up the importance of continuing efforts to improve the product.

The author wishes to express his appreciation to Capt. M. R. Allen, Sgt. Hugh Gray, and Sgt. Chelsia Goodman for their excellent and devoted nursing and clerical help, and without whose services the above study could not have been completed. He would also like to thank Dr. Henry Swan, Professor of Surgery, University of Colorado School of Medicine, and his house staff for their kind cooperation in furnishing us with most of the patient material.

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STUDIES OF THE PHENOMENON OF THE CLEARING OF INFUSED FAT EMULSIONS FROM HUMAN BLOOD AND ITS RELATIONSHIP TO THE FEBRILE REACTION

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IN THE preceding paper, clinical results with an improved fat emulsion for intravenous use in humans were reported The incidence of adverse side reactions as compared with a former preparation was markedly reduced, with the prominent exception of fever which occurs in 29 per cent of the patients The improvement in the reaction rate is ascribed to the removal of impurities" in the soybean phosphatide utilized as the emulsifier in the recent emulsion The fact that the febrile reaction was not affected by the purification of the phosphatide suggested that fever may result from some physiologic mechanism The purpose of this report is to present data which are directly related to this problem and support the concept of "physiologic" fever

The febrile response is a well recognized complication of the administration of intravenous fat emulsions to humans Many investigators have searched for its causation, but none have satisfactorily explained it Such obvious possibilities as pyrogens, particle size, unesterified fatty acid content, and peroxide content have been eliminated as etiologic agents[1] In vivo hemolysis has been incriminated in the past, as well as more recently by Creditor[2] The latter author demonstrated marked increases in mechanical fragility of the red cell in the presence of a fat emulsion and demonstrated hemoglobinemia following an infusion of fat However, the presence of hemoglobin in lipemic plasma, considering the marked increase in mechanical fragility of the red cells, may only indicate trauma coincident with venipuncture and therefore not reflect in vivo hemolysis That this is true is suggested by studies from our laboratory, in which the amount of bilirubin in bile collected from indwelling T tubes in the common duct was measured before and after an infusion of fat[3] No increase in total amount of bilirubin was found following the infusion Furthermore, recent studies by Levenson and Upjohn[4] on red cell survival utilizing the Cr^{51} technique failed to show any effect of multiple fat infusions on the survival curve

Our laboratory has been interested for some time in the problems relating to the clearing of fat from the blood and has performed some studies in normal volunteers on the clearance of intravenous fat emulsions It seemed

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pertinent to extend these observations to a large series of sick persons. Johnson, Freeman, and Meyer,[7] using an emulsion containing 10 per cent olive oil, demonstrated a difference in the clearing rates in a small series of patients with liver, thyroid, or renal disease. It soon became obvious from our studies that there was a great individual variation among patients in their ability to remove the injected fat from the circulatory system. There also appeared to be a direct correlation between the rapidity of clearing and temperature elevation. These and other related observations on clearing are the basis on this report.

MATERIALS AND METHODS

A heterogenous group of hospitalized patients at the Colorado General, Denver General, and Veterans Administration Hospitals in Denver, Colorado, were studied. Eighteen normal volunteers were studied also and are included in the series. No attempt has been made to select patients either for severity or specificity of disease with the exception of a group of patients with cirrhosis of the liver.

The composition of the *"new"* emulsion and *method of administration* were outlined in the preceding paper. Since we were interested in clearing rates, the flow and total time of infusion were carefully controlled and kept as constant as possible. The average time of administration was 216 minutes. The bottle of emulsion contained 90 Gm. of fat; therefore, the flow was approximately 25 Gm. per hour in each subject. Each infusion was begun in the morning before a low-fat breakfast. Lunch was also a low-fat meal and was given after the infusion was completed.

To estimate clearing rate, the following test was devised. The optical density of the subject's serum was measured before, at the end, and eight hours after the beginning of the fat infusion. The optical density (O.D.) was measured with a Coleman Jr. Spectrophotometer at 700 $m\mu$, using water as the blank. The bloods were kept at 40° F. temperature during the test day and read the morning following the day of infusion.

Body temperature of the recipient was measured hourly for a period of twelve hours. Rectal temperatures were recorded whenever possible. A febrile reaction has been defined as a rise in temperature of at least 2° F. above 99.6° F. rectally, or 2° F. over the control temperature, if that figure is above "normal."

Soon after the start of the study, it became apparent that there was a great variability among patients in the rapidity with which their blood cleared of the infused fat. At first, arbitrary figures for the optical density at the various sampling times were set in an effort to categorize the patients according to fast, slow, or normal clearing. Thus, those patients whose serum had an optical density above 1.0 at the end of the infusion and had returned to preinfusion levels by 8 hours were considered normal clearers. If the plasma remained lipemic after 8 hours, the subject was said to be a slow clearer; if the optical density was below 1.0 at the end of the infusion, then he was said to be a fast clearer. These arbitrary criteria were based on previous experience with normal volunteers. However, it became obvious that delineation into fast, slow, or normal clearing patterns was not practical, since some of our volunteer subjects did not fit the "normal" pattern. It seems preferable, therefore, to refer to actual optical density readings rather than arbitrary limits, reminding the readers that an optical density figure above 0.3-0.4 represents grossly lipemic serum.

Optical density is a simple, convenient means of measuring lipemia. Other techniques, such as chylomicron counts or nephelometry, have well-recognized limitations and, in the case of the former, are time consuming as well as fraught with error.[6] Optical density measures transmission of light across a given path, so that any particles present which reflect or diverge the path of light will affect the optical density. Thus, the latter is proportional to the concentration of particles; in our case, concentration of fat particles. Since all of the fat administered in the emulsion is in the form of chylomicrons, the clearance of these fat particles, therefore can be followed with optical density measurement. This assumes of course that most of the fat leaves the blood stream as chylomicrons rather than first becoming solubilized to lipoprotein. The correlation between optical density and total fatty acids following an infusion of fat emulsion is shown in Fig 1. The sigmoid curve reflects the inefficiency of the Coleman spectrophotometer at the upper level and the individual fasting variations in soluble fat at the lower level. However, in the ranges in which we are most interested, that is, O D from 0.3 to 1.5, there is a moderately good straight line relation between total fatty acids and optical density. Interfering pigments such as bilirubin and hemoglobin, will affect the readings to a small insignificant degree.

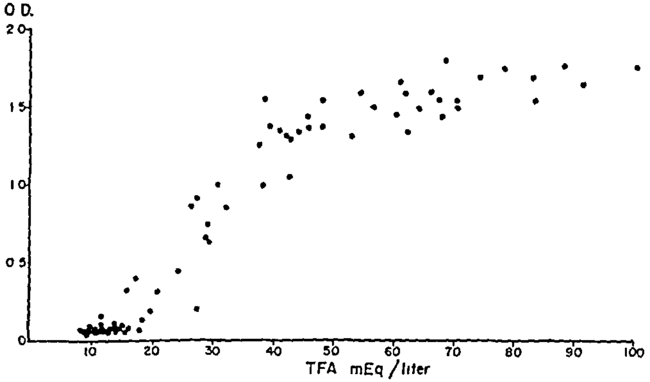

Fig 1.—The relationship between the optical density of the whole serum and the total fatty acids of the same whole serum

RESULTS

In all, 225 clearing studies have been performed on 120 subjects infused with the "new" emulsion. The large amount of data accumulated does not permit detailed presentation of individual records or results. An insight into some of this information can be gained from the accompanying graphs. Several means are available to assess the rate of clearing in an individual and make comparisons among individuals. Since a constant amount of fat is infused at a constant rate and the clearing process is a dynamic one, the optical density readings at comparable times can be used. Thus in Fig 2 the optical density of serum at 8 hours is plotted against the highest rectal temperature reading in the 12 hour period of the experiment for 95 subjects receiving their first infusion. With two exceptions significant fever did not occur in those subjects whose serum was lipemic (O D > 0.3) after 8 hours. Conversely, those patients who develop a febrile reaction to the intravenous fat have essentially clear serum at 8 hours although fever does not invariably result from rapid clearing. The same relationship is obtained when the optical density at the end of infusion is plotted against temperature as in Fig 3. In both instances the correlation is highly significant ($P < 0.01$). Since clearing is a dynamic process, the change in optical density during infusion and the per cent decline in optical density after infusion when compared with temperature rise, likewise show a highly significant correlation ($P < 0.01$).

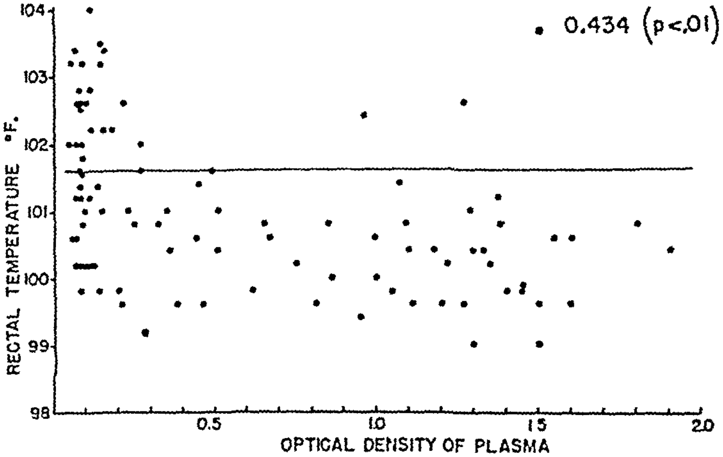

Fig. 2.—Relation between the optical density of the plasma 8 hours after the start of an infusion of 600 ml. of Lipomul and the highest rectal temperature in the 12-hour post-infusion period in 95 subjects receiving their first infusion.

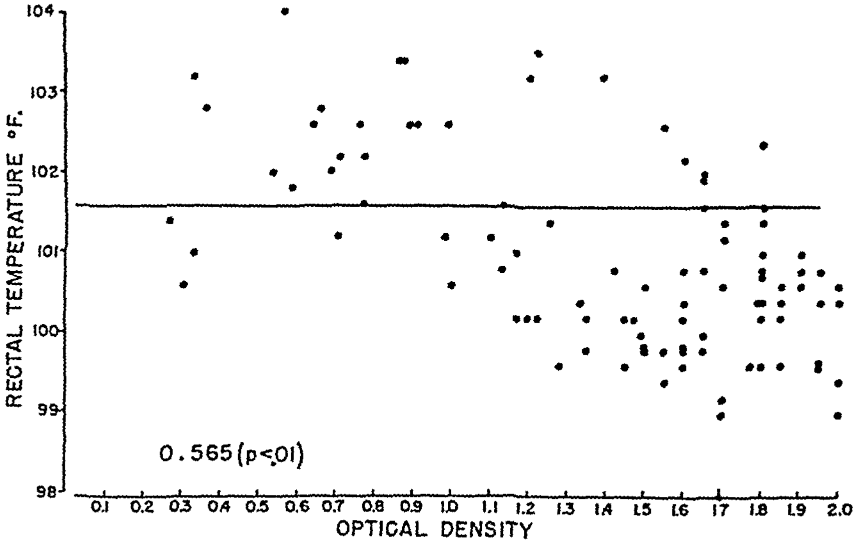

Fig. 3.—Relationship between the optical density of the plasma immediately after the end of an infusion of 600 ml. of Lipomul and the highest rectal temperature in the 12-hour postinfusion period in 95 subjects receiving their first infusion.

When the data are analyzed for 168 infusions, which represent repeat infusions in some subjects, the correlation between the optical density at the end of infusion and at 8 hours and the highest rectal temperature again is significant at the 1 per cent level of probability. However, if one analyzes the results for the 73 repeat infusions alone, significance is not attained (Fig. 4).

Some interesting observations have been made concerning the clearing pattern on repeat infusions in the same individual. Thirty such studies have been made, of which a representative number have been depicted in Fig. 5.

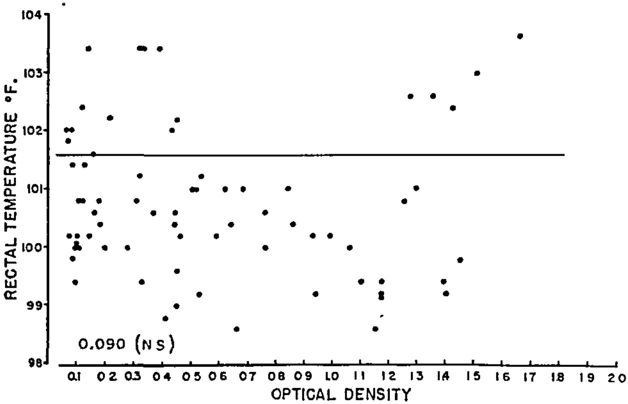

Fig 4.—Relationship between the optical density of the plasma 8 hours after the start of an infusion of 600 ml of Lipomul and the highest rectal temperature in the 12 hour postinfusion period of 73 repeated infusions

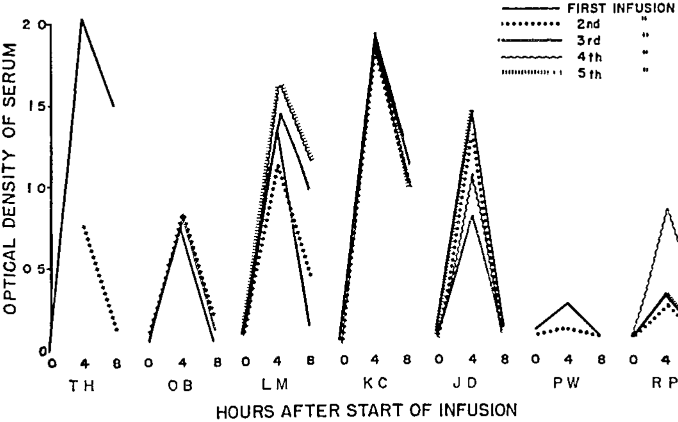

Fig 5.—Representative examples of individual patients' clearing test following 600 ml. of Lipomul on successive days

It is clear from this sample that the previously mentioned variation in first infusions is also present in successive ones. There is no regular sequence of clearing in each individual. The normal individuals, as well as patients been found in human blood in appreciable amounts only as a result of the injection of heparin or a like substance. Cleland and Iacono[12] in our laboratory have measured the amount of clearing factor activity present in the blood of some of the subjects following an infusion of fat, utilizing a method which measures glycerol production in vitro over a 4-hour period. None of the subjects was heparinized. They have found an insignificant amount of enzyme present which could account for the hydrolysis of approximately 5 per cent of the fat which is cleared. Thus, it would appear that clearing factor enzyme, better referred to as lipoprotein lipase, plays a very insignificant role in the actual clearing of the blood.

The role of the reticuloendothelial system in the clearing process has been extensively studied by others.[13] Histologic scrutiny of tissues of animals immediately following the infusion of a fat emulsion has shown fat globules in macrophages of the spleen and Kupffer cells of the liver.[14] However, the experiments by Waddell and co-workers[13] in rats showed very clearly that blockage of the reticuloendothelial system with India ink or trypan blue did not affect the clearing of an injection of fat emulsion from the blood, but that evisceration and, in particular, hepatectomy, grossly slowed the clearing.[15]

The studies herein reported do not shed much light upon the role of the liver in the complex clearing mechanism. Of the 11 cirrhotic patients in this series, all but one were slow clearers. However, so were most of the normal volunteers. This is in distinct contrast to the studies of Johnson and associates,[5] in which the cirrhotics cleared faster than normal. Unfortunately, none of our patients were suffering from acute liver disease or intoxication, so that the observations by Scott and Vars[16] that chloroform intoxication of the liver increased the clearing of fat could not be verified.

Recently, interest has developed in the effects of aging and, in particular, atherosclerosis, on the disappearance rate of lipemia produced by an oral fat meal. Necheles and co-workers[17, 18] reported that aged individuals clear their blood of ingested fat more slowly than younger persons, although White, Ralston, and Carne[19] could not corroborate this effect but found instead a correlation between bed rest and slow clearing. Moreton[20] first called attention to the fact that patients with atherosclerosis clear more slowly than those without this affliction. These observations have been confirmed by Pomeranze, Beinfield, and Chessin[21] in the obese and atherosclerotic patient, and by Schwartz, Woldow, and Dunsmore[22] in patients with myocardial infarction. Utilizing I^{131}-labeled neutral fat by mouth, Thannhauser and Stanley[23] demonstrated slow clearing in patients with idiopathic hyperlipemia as compared to normal subjects. All of these observations are complicated by the process of fat absorption, so that elevated or prolonged blood lipid level after a fat meal may not, of necessity, reflect the activity of the clearing mechanism, but rather an absorptive phenomenon.

Our study was not designed to investigate any specific disease. However, because of the interest in the possible etiologic relationships between lipemia and various disease states, a review of our case material is presented in Table I with these thoughts in mind. Because of the arbitrary categorization into slow, intermediate, and fast clearing patterns and the small number of subjects in certain areas of interest, it is only possible to derive trends or suggestions from these data. However, it is of interest to note that as weight increases, slowness of clearing likewise becomes more common. There is no apparent correlation between the presence or absence of diastolic hypertension and the pattern of clearing. The converse of the results obtained with an oral fat load in various age groups was found in our studies, wherein increased clearing was associated with increasing age. The types of clearing

TABLE I. THE RELATION OF VARIOUS PHYSIOLOGIC PARAMETERS AND DISEASE STATES AND THE PATTERN OF CLEARING OF THE BLOOD FOLLOWING AN INFUSION OF A FAT EMULSION

		CLEARING PATTERN					
	TOTAL	INTERMEDIATE*		SLOW†		FAST‡	
Weight	NO.	NUMBER	(%)	NUMBER	(%)	NUMBER	(%)
Less than 100 pounds	8	6	(75)	2	(25)	0	(0)
100-150 pounds	76	27	(35)	28	(36)	21	(29)
150-200 pounds	14	6	(43)	6	(43)	2	(14)
Over 200 pounds	1	0	(0)	1	(100)	0	(0)
Blood Pressure							
Diastolic above 90 mm. Hg	28	16	(57)	8	(28)	4	(15)
Diastolic below 90 mm. Hg	77	28	(36)	29	(37)	20	(27)
Age							
20-40 years	21	7	(33)	12	(57)	2	(11)
40-60 years	39	11	(28)	20	(51)	8	(21)
60-80 years	54	28	(52)	11	(20)	15	(28)
Clinical Diagnosis							
Hyperlipemia	1	0	(0)	1	(100)	0	(0)
Diabetes mellitus	2	1	(50)	1	(50)	0	(0)
Coronary occlusion	5	1	(20)	4	(80)	0	(0)
Stroke	7	3	(43)	4	(57)	0	(0)
Arteriosclerotic heart disease	9	4	(44)	1	(12)	4	(44)
Generalized arteriosclerosis	17	8	(47)	4	(23)	5	(30)
Family history of any of the above	21	6	(28)	11	(52)	4	(20)
Cirrhosis of liver	11	0	(0)	10	(91)	1	(9)
Normal volunteers	18	6	(33)	12	(66)	0	(0)

*Intermediate = O.D. above 1.0 at end of infusion, below 0.3 after 8 hours.
†Slow = O.D. above 1.0 at end of infusion, above 0.3 after 8 hours.
‡Fast = O.D. below 1.0 at end of infusion, below 0.3 after 8 hours.

patterns observed in the various disease entities are confusing and probably reflect the complex nature of the clearing phenomena. These patients were suffering from either complications of their primary illness or some other unrelated affliction which undoubtedly affects the clearing pattern and leads to the vagaries noted. One would like to study the various illnesses in an uncomplicated form, and, even then, the inborn variation in the physiologic phenomena of clearing may cloud the issue.

SUMMARY AND CONCLUSIONS

1. A clearing test has been devised which measures the rapidity at which the blood is cleared of infused fat. It has been applied to the study of 120 subjects receiving 225 infusions of an emulsified fat preparation.

2. Twenty-nine per cent of the subjects receiving the fat developed a significant elevation of body temperature.

3. There is a highly significant correlation between the rapidity of clearing and the febrile response.

4. These results are interpreted as evidence that the febrile response to intravenous fat therapy may be due to rapid oxidation of the fat with resultant endogenous heat production and should be considered a physiologic reaction in susceptible individuals. The nature of the fever is such that it is not considered a contraindication to the use of fat emulsion for parenteral alimentation.

5. The possible reasons for the great variability in clearing patterns between individuals, as well as between successive infusions in the same individual, are discussed. Until the basic physiologic mechanism regulating fat transport in the blood is better understood, the vagaries will remain obscure.

6. Lines of future investigation are cited, and it is hoped that the fat emulsion will prove a useful tool in future studies of fat metabolism.

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THE EFFECTIVENESS OF MEPROBAMATE AND LOBELINE AS SMOKING DETERRENTS

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Ever since its introduction, the use of tobacco has been criticized by various groups for varied reasons. The pressure on a smoker to quit has been increased of late, chiefly because of reports linking tobacco use with a higher incidence of bronchiogenic carcinoma. There is also some concern regarding the role of tobacco in peptic ulcer, coronary artery disease, peripheral vascular disease, and other bronchopulmonary disease. Disregarding for the moment the part that tobacco plays in these diseases, let us consider what can be done to help the patient who wants to stop smoking or who has been advised by his physician to quit.

In 1936, Dorsey reported encouraging results with the use of lobeline.[1] Wright and Littauer later reported that the side effects of lobeline were such as to preclude its general use.[2] Rapp and Olen recently published a study which indicated that they had circumvented the side effects by combining lobeline with an antacid mixture; they stated that the preparation was very effective in reducing the desire to smoke.[3] Another preparation for the relief of withdrawal effects has been reported; it contains $\frac{1}{20}$ grain benzocaine flavored with saccharin, extract of liquorice, powdered ginger, and oils of anise, wintergreen, peppermint, coriander, and cloves.[4] However, it is our opinion that this study was not sufficiently controlled to warrant prescribing this aromatic preparation. Other methods have been proposed from time to time, but none have been successful.

Johnston states that the only way to stop smoking is by not smoking again.[5] This Spartan dictate is often ignored by patients, who feel that they need something more to help them through the withdrawal period. Johnston feels that there are two components involved in the desire to continue smoking; the first is a pharmacologic craving which is of high intensity but of short duration, reaching a peak 24 to 36 hours after stopping, while less intense psychologic urges may persist for years. Obviously, a drug which could help prevent the patient from starting to smoke again in the first few weeks would be very useful.

The popularity of the ataraxic drugs seemed to offer a new attack on this problem. Since most cigarette smokers state that a smoke "relaxes" them, and

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The drugs used in this study were furnished by Wallace Laboratories (Miltown) and Compana Sales Corporation (Bantron). A grant from Wallace Laboratories was used to defray expenses.

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since the ataraxics also "relax" the patient, we felt that the effect of a tranquilizer might logically be expected to reduce the need for cigarettes. We also wished to check the efficacy of the lobeline antacid mixture.

PROCEDURE: PART I

Thirty-three young, adult medical students were used, each of whom stated that he was in good health. These subjects were divided into 4 groups; the drug schedule was so arranged that the order of drugs was different for each group, but each subject still received all 3 preparations. The tests were run in 3-week cycles, the first control week was a determination of the number of cigarettes smoked normally for a 7-day period while the second week was a determination of the cigarettes smoked while taking one of the drugs. There followed a third recovery week wherein the subject took neither a preparation nor kept a record of the cigarettes smoked (Table I).

TABLE I. SCHEDULE OF DRUG ADMINISTRATION IN PART I

SUBJECTS	WEEK OF TEST							
	1	2	3	4	5	6	7	8
1–9	Control	Placebo	Recovery	Control	Bantron	Recovery	Control	Miltown
10–18	Control	Bantron	Recovery	Control	Miltown	Recovery	Control	Placebo
19–27	Control	Placebo	Recovery	Control	Miltown	Recovery	Control	Bantron
28–33	Control	Miltown	Recovery	Control	Bantron	Recovery	Control	Placebo

The drugs tested were meprobamate, and a commercial preparation known as Bantron, which was stated to be identical with that used by Rapp and Olen. A sugar placebo was included, and all drugs were powdered and placed in identical No. 0 pink capsules to be taken 3 times daily. A capsule contained either (1) 400 mg. meprobamate, (2) the equivalent of 1 tablet of Bantron, or (3) sugar. The subjects were aware of the fact that they might be given a placebo, but they were not told which preparation they were receiving at any given time. They were instructed to make no attempt to influence their smoking level, but merely to have a cigarette when they felt they needed one. It was felt that this procedure would measure any depressant effect on smoking, and would tend to eliminate the factor of "will power."

The subjects were told that we had reason to believe that the drugs they were being given would probably reduce their cigarette consumption. The methods used for evaluation were those of the double-blind technique.

RESULTS: PART I

The thirty-three subjects had smoked an average of 8½ years, and at the time of the test were smoking around 16 cigarettes per day. The results were calculated for the group as a whole in terms of the average number of cigarettes per day per subject. In analysis, it was observed that neither meprobamate nor the commercial lobeline preparation was significantly more effective in reducing

TABLE II. AVERAGE NUMBER OF CIGARETTES SMOKED PER DAY PER SUBJECT FOR 33 SUBJECTS

	DAY OF TEST						
	1	2	3	4	5	6	7
Control	16.8	17.9	18.3	17.9	17.6	16.1	15.8
Bantron	16.7	17.2	16.0	17.4	17.4	15.4	16.1
Control	17.8	16.9	18.1	18.4	19.2	16.0	17.3
Placebo	16.9	16.2	16.9	17.2	16.6	15.9	15.0
Control	17.5	17.0	17.4	17.4	17.9	17.1	16.2
Miltown	16.1	17.0	17.4	17.9	17.1	15.5	15.6

the smoking levels than was the sugar placebo (Table II). The group was then subdivided into subgroups of those smoking between 5 and 14.9 per day, between 15 and 24.9 per day, and over 25 per day and the data re-evaluated. The results of this analysis did not differ significantly from that of the group as a whole. We are at a loss to explain the great discrepancy between our results and those of Rapp and Olen.[3]

PROCEDURE: PART II

It could be argued that we were not measuring the effect of the drugs to replace cigarettes by the above procedure. Hence, we obtained volunteer subjects who expressed a strong desire to stop smoking. These subjects were arbitrarily divided into two groups, one of which was given capsules containing 400 mg. meprobamate, and the other given indistinguishable placebo preparations. The subjects were instructed to take a capsule whenever they felt the withdrawal symptoms start to appear, but not to take over 8 capsules per day. They were further told that the drug would be effective in reducing the desire for a cigarette. When the tests were completed, the groups were as follows:

Miltown Group: 6 men and 3 women, smoking for an average of 9.3 years and smoking an average of 17.5 cigarettes per day prior to the test

Placebo Group: 7 men, smoking for an average of 8.4 years and smoking an average of 21.6 cigarettes per day just preceding the test

RESULTS: PART II

No significant difference was noted between the two groups, either by success in quitting or by their impression of the effectiveness of the drug. It was of especial interest that only one of the subjects given the placebo felt that this had not helped him (Table III).

Table III. Composition of Test Groups and Results (Part II)

	MEN	WOMEN	AVERAGE YEARS SMOKING	AVERAGE DAILY CIGARETTE USE	SUBJECTS CEASED SMOKING	SUBJECTS FELT DRUG WAS OF VALUE
Miltown	6	3	9.3	17.5	3	8
Placebo	7	0	8.4	21.6	3	6

DISCUSSION

In spite of the favorable reports in the literature on the effectiveness of lobeline, we were unable to demonstrate that the preparation used has any effect on cigarette consumption. Similarly, although the use of a tranquilizing drug would appear indicated, the one used in this study, given in the manner described, had no effect. Since the subjects were told to take a capsule whenever they first noticed the symptoms of withdrawal start to appear, we feel that the time necessary for the drug to be released from the capsule, absorbed, and exert its effect may have been excessive. In other words, the symptoms may have become intolerable before the drug took effect. A faster acting drug, or perhaps a high "prophylactic" daily dose, might be more effective.

The present treatment of tobacco withdrawal effects would seem to be psychological; we know of no currently used pharmacologic agent which has a clearly effective action. A procedure to be used when dealing with a patient who must or desires to stop smoking might be as follows: first, make sure that he really wants to quit; if this motivation is lacking, no success can be expected. Second, reinforce that desire by telling him of studies incriminating tobacco as a pathogenic agent. And finally, support him in his endeavor by other psychologic measures, including the use of sugar capsules if deemed necessary.

SUMMARY

In a study of the smoking habits of thirty-three medical students no effect was noticed upon administration of meprobamate or a commercial buffered lobeline preparation.

In a study involving 15 medical students who stated they wished to stop smoking, no objective or subjective difference was noted between the group receiving meprobamate and those receiving a placebo. However, 6 out of 7 subjects felt that the placebo had helped them to resist the desire to smoke.

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DETERMINATION OF AROMATIC ACIDS AND PHENOLS IN THE URINE OF WHITE AND NEGRO MALE ADULTS

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THE possible relationship of tyrosine and phenylalanine metabolism to phenylacidurias in tyrosinosis, mental disease, vitamin C and folic acid deficiencies, etc., and also of the enzymatic oxidation of tyrosine by tyrosinase and dopa-oxidase to aromatic acids and the melanin pigments still remains obscure.

As a possible tool in studying these conditions, microbiologic methods were developed for the assay of phenyllactic, *p*-hydroxyphenyllactic, and indole-3-lactic acids in urine. In addition, ether extracts of these urines were analyzed for total volatile phenols and aromatic hydroxy acids by previously described methods[1, 2] which utilize the Folin-Ciocalteau reagent. Perhaps the Negro, due to the presence of large amounts of dopa-oxidase in the skin, might excrete increased amounts of phenylaciduric or phenolic material. Preliminary experiments had encouraged us in this belief.[3] Also, recent work indicates that Chinese, for example, excrete significantly greater quantities of amino acids in urine than Caucasoids and that diet is not a principal factor in this difference.[4]

MATERIAL AND METHODS

Twenty-four-hour specimens of urine were collected for 2 consecutive days from apparently healthy Negro and white male adults. Forty milliliter aliquots of each urine were acidified with 10 N sulfuric acid to pH 0 and refluxed for one hour. The hydrolysates were adjusted to 200 ml. at pH 10 with dilute sodium hydroxide and then extracted continuously with redistilled (peroxide-free) ether for 24 hours.[1, 5] Each extract was analyzed for the volatile phenol fraction with the Folin-Ciocalteau reagent as previously described.[2] The residue in each extractor was acidified with 2 ml. of 10 N sulfuric acid and extraction continued for 24 hours. This extract contained the aromatic acid fraction. The ether was volatilized and the aqueous residue neutralized to pH 6.9 and diluted to 25 ml. The total aromatic hydroxy acid content was also determined with the Folin-Ciocalteau reagent.[2] Then, individually, phenyllactic, *p*-hydroxyphenyllactic, and indole-3-lactic acids were determined with *Lactobacillus casei* (ATCC No. 7469) as described below.

Our microbiologic assay procedures, media, cultures, inocula, etc., have been described elsewhere.[1, 6] The basal media were premixed crystalline preparations of nutrients.† Each preparation was nutritionally complete for *L. casei* except for the specific amino acid whose hydroxy analog was being assayed. A weighed quantity of each medium was freshly dissolved in distilled water and adjusted to pH 6.9.

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†Manufactured by the H. M. Chemical Company, Santa Monica, California.

Pyrex tubes (18 × 150 mm) were immersed in chromic acid cleaning solution, washed with water 10 times, autoclaved with distilled water, and then dried A 1 ml quantity of the appropriate basal medium, depending upon the amino acid analog being assayed, was added to each tube A 0 5, 1 0, or 2 0 ml aliquot of a sample was added and the contents of each tube were made up to 5 ml with distilled water Each sample was assayed in replicate

The tubes were covered with aluminum caps, autoclaved at 15 pounds pressure for 15 minutes, cooled inoculated, and then incubated for 72 hours at 34° C Since urine extracts are frequently colored the acidity produced by the organism was titrated electrometrically[7] With each urine assay, standard growth curves were developed simultaneously with phenyllactic acid, p hydroxyphenyllactic acid, and L tryptophane (indole 3 lactic acid was not available) If any keto analogs of these amino acids were present in the urines, they would be destroyed by the drastic acid hydrolysis[8]

When 200 to 20,000 mcg of each hydroxy analog was added to urine and hydrolyzed at pH O, extracted, and assayed as described above, recovery values ranged from 86 to 102 per cent The addition of 2 mg of Tween 40 per tube (data omitted), to eliminate the inhibitory action of any oleic acid present in the urine extracts, did not change the results of the assay

Previous work from this laboratory demonstrated that significant changes in the concentration of urinary phenolic bodies were not observed between human subjects on high and low protein diets This effect was observed only when protein was rigidly eliminated from the diet[9] Therefore no check on food intake was made except by a total urine nitrogen determination Each subject was warned repeatedly to avoid medicines or drugs

RESULTS

The results of the analysis of two consecutive 24 hour urine samples from 10 individuals are reported in Table I It is obvious that the values for the various urinary constituents, especially the microbiologically determined aromatic acids, vary widely from subject to subject although the two consecutive specimens in each case show some degree of uniformity These variations, however, are not surprising in view of the work of Williams and his colleagues who concluded that every individual possesses a distinctive metabolic personality or pattern and that values for various urinary constituents obtained from different individuals often differ by as much as several hundred per cent[10]

p Hydroxyphenyllactic acid was present in small amounts ranging from 318 to 1,475 mcg, average 860 mcg, for the urines from the whites and 166 to 1,465 mcg, average 699 mcg, for those from the Negroes Indole 3 lactic acid was also present in small amounts, ranging from 108 to 486 mcg, average 341 mcg, and 193 to 821 mcg, average 500 mcg for the same urines The amount of phenyllactic acid ranged from insignificant traces to quantities not exceeding 406 mcg per 24 hour samples

The total aromatic hydroxy acids determined with the Folin Ciocalteau reagent on ether extracts and calculated as p hydroxyphenylacetic acid ranged from 155 to 450 mg, average 253 mg, and 174 to 258 mg, average 219 mg for the two groups of urine samples Here relative variations between urines are somewhat smaller Thus, the 3 amino acid analogs determined microbiologically constitute less than one per cent of the total aromatic hydroxy acid fraction determined chemically and calculated as p hydroxyphenylacetic acid

It appears obvious, however, from the good recovery values obtained for the microbiologically determined aromatic acids, that the relatively larger quantities of other aromatic acids in the urines did not interfere with the utilization of the amino acid analogs by *L. casei.* Previous work[7] from this laboratory showed that the various aromatic acids normally present in urine are relatively poor inhibitors of the metabolic conversion of phenyllactic acid to phenylalanine by *L. casei.* In this respect, however, mandelic acid proved to be a potent inhibitor.

TABLE I. AROMATIC ACIDS AND PHENOLS IN URINE FROM WHITE AND NEGRO MALE ADULTS*

SUBJECT	RACE	VOLATILE PHENOLS (MG.)	AROMATIC HYDROXY ACIDS (MG.)	PHENYL-LACTIC ACID (MCG.)	p-HYDROXY-PHENYL-LACTIC ACID (MCG.)	INDOLE-3-LACTIC ACID (MCG.)	TOTAL NITROGEN (GM.)
K.	White	117	228	78	469	254	14.1
K.	White	117	256	I†	516	258	14.9
V.	White	91	182	69	318	486	11.0
V.	White	125	165	I	354	267	11.6
H.	White	224	437	I	1,131	108	15.7
H.	White	302	450	I	1,151	200	15.9
W.	White	172	255	264	1,475	395	16.2
W.	White	127	204	I	1,400	467	11.4
S.	White	119	155	406	922	601	12.0
S.	White	136	196	117	860	371	12.0
Max.		302	450	406	1,475	486	15.9
Min.		91	155	I	318	108	11.4
Ave.		153	253	--	860	311	13.5
M.	Negro	147	226	156	547	332	11.4
M.	Negro	166	209	234	719	352	13.6
P.	Negro	163	196	I	166	193	10.3
P.	Negro	146	174	I	183	414	9.1
Q. J. R.	Negro	161	250	116	679	475	11.0
Q. J. R.	Negro	106	258	150	395	197	10.5
R.	Negro	107	237	I	850	795	17.4
R.	Negro	113	226	I	1,015	821	14.7
Q.	Negro	116	225	289	960	700	11.4
Q.	Negro	118	192	312	1,465	720	10.7
Max.		163	258	312	1,465	821	17.4
Min.		106	174	I	166	193	9.1
Ave.		134	219	--	698	500	12.0

*All values are based on 24-hour urine samples and volatile phenols as p-cresol and aromatic hydroxy acids as p-hydroxyphenylacetic acid.

†Insignificant trace

Urinary volatile phenols, probably mainly p-cresol,[11] ranged from 91 to 302 mg., average 153 mg., and 106 to 163 mg., average 134 mg., calculated as p-cresol per 24 hours, for the urines from the white and Negro subjects.

While, therefore, individual urines show wide variations in the concentration of the various aromatic acids and phenolic bodies, no characteristic or outstanding racial difference between the two groups of urines appears to be present.

SUMMARY

Methods are described for the microbiologic determination of phenyllactic, p-hydroxyphenyllactic, and indole-3-lactic acids in urine. The volatile phenolic

and aromatic hydroxy acid fractions were determined chemically with the Folin Ciocalteau reagent While individual urines usually showed wide variations in these constituents, no obvious chemical difference between the urines from the white and Negro subjects appeared to be present

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PLASMA 17-HYDROXYCORTICOSTEROIDS IN HYPERFUNCTION, SUPPRESSION, AND DEFICIENCY OF ADRENAL CORTICAL FUNCTION

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CONSIDERABLE advances have been made during the last decade in the understanding and evaluation of adrenocortical function in man. The determination of the 17-ketosteroid concentration in the urine has served as a useful tool in the clinical appraisal of endocrine abnormalities for nearly 20 years. The availability of adrenocorticotropin (ACTH) and the introduction of new methods for the estimation of steroids in body fluids, however, has made it possible to evaluate adrenocortical function more reliably. An important advance was the introduction by Thorn and associates[1] of the response of blood eosinophils to the administration of ACTH as a test of adrenocortical function. Subsequently, Thorn and co-workers[2] reported that the changes in the urinary excretion of 17-ketosteroids and 17-hydroxycorticosteroids were more direct indices of adrenocortical function. Recently, the development of methods for the determination of free blood 17-hydroxycorticosteroid (17-OHCS) levels has made it possible to study more precisely changes in adrenocortical activity and in the metabolism of 17-OHCS.[3, 4] The measurement of free plasma 17-OHCS reveals the concentration of corticosteroids before they are extensively metabolized, conjugated, and excreted in the urine.[3, 4]

In this paper we wish to present data on plasma 17-OHCS levels and their changes during ACTH or steroid administration in: (a) patients who had been on prolonged steroid therapy; (b) patients with adrenal insufficiency; and (c) patients with Cushing's syndrome. In addition, the metabolism of C^{14}-labeled cortisol (hydrocortisone) was studied in some of the subjects.

METHODS AND MATERIALS

Plasma 17-OHCS levels were determined in the early part of the study by the method of Nelson and Samuels[3, 4] and, more recently, by the method of Silber and Porter[5] as modified by Eik-Nes.[6]

Plasma 17-OHCS levels on patients who had been on prolonged cortisone (or cortisol) therapy were determined on bloods drawn following cessation of such therapy. The last dose of steroid was taken not later than 6 P.M. and the blood samples were obtained between 9 and 10 A.M. of the following day. The ACTH tests and the method of

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administration of various steroids have been described previously[7, 8] The methods for the administration of 4 C^{14} cortisol and determination of radioactivity have been described in a previous publication[9] Patients with known Addison's disease were taken off their cortisone or cortisol therapy for a minimum of 5 days before the test but were maintained on DOC therapy

RESULTS

1 *Patients Studied After Abrupt Cessation of Steroid Administration* — The levels of plasma 17 OHCS over a 6 hour period (9 A M to 3 P M) in a group of 12 patients who had been on prolonged cortisol or cortisone therapy, followed by abrupt cessation of the therapy the day before the blood samples were obtained, are compared in Fig 1 with the values obtained in a group of 16 normal subjects The mean values for the patients who had been on steroid therapy are much lower than those of the control group Most of the values (0 to 4 μg/100 ml of plasma) in the treated subjects were below the level of dependability of the method employed and are difficult to determine accurately by the method used[3, 4] Some of the patients had values well within the normal range, even though these subjects had been on high doses of steroids for prolonged periods of time The gradual decline of 17 OHCS levels in the control group reflects the normal diurnal variation of these 17 OHCS[10]

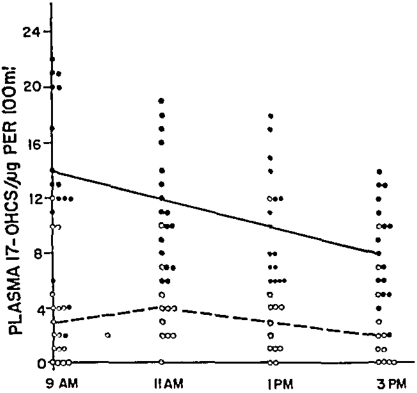

Fig 1 —Plasma 17 OHCS levels in patients (open circles) following abrupt cessation of long term steroid therapy the day before the sampling and in normal subjects (closed circles) from 9 A M to 3 P M The solid and broken lines represent the means of the plasma 17 OHCS in the normal subjects and in the steroid treated patients respectively

In order to evaluate adrenocortical capacity in patients who had been on prolonged steroid therapy, a standard ACTH test was performed by infusing 40 I U of ACTH intravenously over 6 hours[7] In Fig 2 are shown the changes in plasma 17 OHCS levels during the infusion of ACTH It is apparent that 5 of the patients had a subnormal response The 17 OHCS rose normally in one patient, a 66 year old woman with a disease suggestive of lupus erythematosus who had been on oral cortisone acetate therapy (50 to 150 mg every day) for at least a year. In Fig. 3 are shown the changes in plasma 17-OHCS levels in a male patient (L. C.) with rheumatoid arthritis given ACTH on 4 successive days following cessation of long-term cortisone acetate therapy.

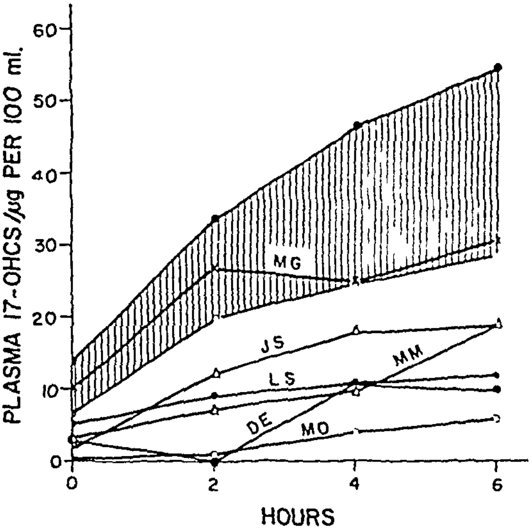

Fig 2—Changes in plasma 17-OHCS levels during the infusion of 10 units of ACTH in 6 subjects following abrupt cessation of prolonged steroid therapy. Shaded area represents range of response in normal subjects

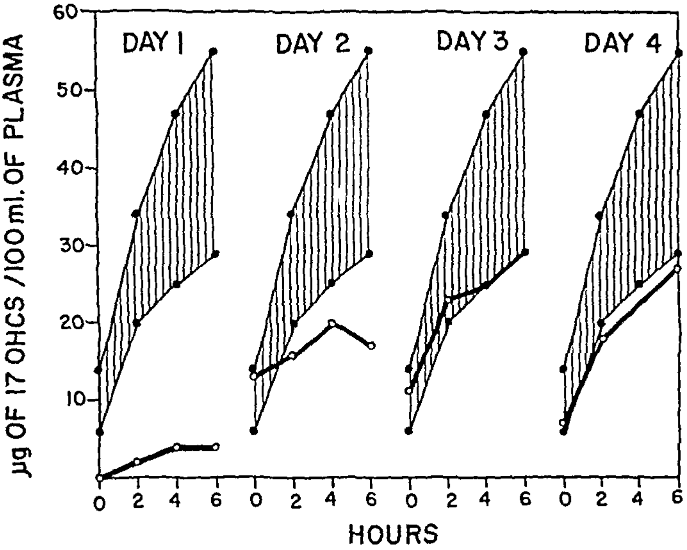

Fig 3—Changes in plasma 17-OHCS of Subject L C (40-year-old male) a patient with rheumatoid arthritis treated with cortisone acetate (250 mg daily orally) for 6 months On four successive days, following sudden discontinuation of the steroid therapy, ACTH (40 units) was given intravenously over 6 hours and the changes in plasma 17 OHCS were determined The shaded areas represent the range of normal response

On the first day the response was definitely subnormal, similar to the 5 patients in Fig. 2, on the second day it was slightly more adequate although still not normal, and on the third and fourth days the responses approached normal. It should be noted that the changes in the plasma 17 OHCS response in Patient L. C. were probably influenced by the daily administration of ACTH.

One patient (W. N.), a 32 year old woman with acute leukemia, had been on 200 mg. of cortisone acetate for one month, followed by gradual discontinuance of the steroid therapy over a period of several days, with the last dose of 25 mg. cortisone acetate being taken on March 31. On April 1 the patient's plasma 17 OHCS levels were 7 μg at 9 A.M., 9 μg at 10 A.M., and 4 μg at noon. The evening of that day the patient developed severe chills, fainted, and was hospitalized for septicemia. On April 2 her plasma 17 OHCS were 42 μg at 9 A.M., 39 μg at 10 A.M., and 27 μg at noon. In this patient rapid recovery of adrenocortical capacity under stress occurred even after steroid therapy of several weeks' duration.

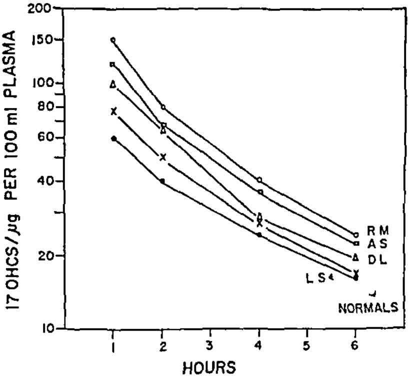

Fig. 4.—Clearance of 17 OHCS from plasma following the intravenous injection of 50 mg. of cortisol into subjects following abrupt cessation of long term cortisone or cortisol therapy. The results are compared to the average of normal subjects. The control plasmas of the patients did not contain any demonstrable 17 OHCS levels.

One possible explanation for the low plasma 17 OHCS levels in patients after long term administration of steroid compounds is that the prolonged administration of steroids might alter the metabolism of 17 OHCS. In order to test this possibility, 4 patients who had been on long term, high dose therapy of cortisone acetate received 50 mg. of cortisol intravenously on the day following abrupt cessation of treatment, and the clearance of 17 OHCS from their plasma was determined. The results are shown in Fig. 4. In none of the 4 subjects were concentrations of 17 OHCS determinable in the control plasma samples. The clearance rates in the patients were similar to those of normal subjects, even though in 2 patients (R. M. and A. S.) the concentrations were significantly higher than those seen in normal subjects. The amount of cortisol administered to the patients and the normal subjects was approximately twice the physiologic daily requirement, however, and although the results do not support the hypothesis of altered metabolism, they do not disprove it. To pursue this proposition further, tracer amounts of C^{14}-labeled cortisol (1 μc/245 μg) were given to 2 patients following sudden cessation of

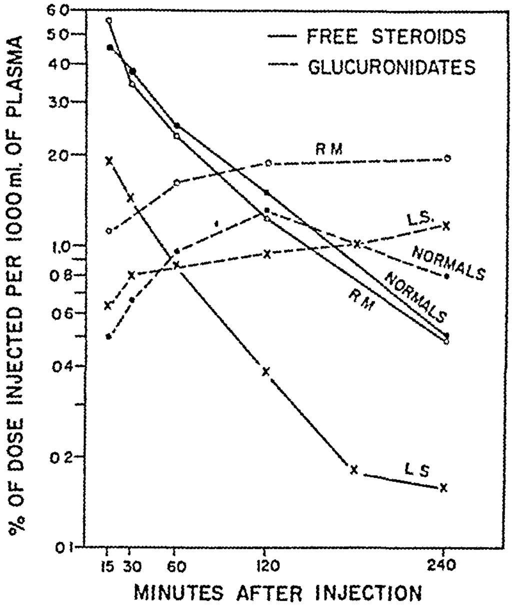

Fig. 5.—Radioactivity in the plasma free-steroid fraction and in the glucuronidate fraction of normal subjects and two patients, who had been on prolonged steroid therapy, following the injection of C^{14}-cortisol. Patient L. S. had the nephrotic syndrome with generalized, severe edema complicating generalized lupus erythematosus at the time of the study.

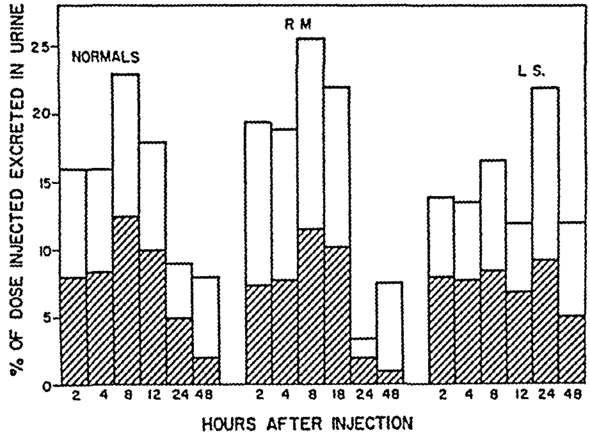

Fig. 6.—Urinary radioactivity following the injection of C^{14}-cortisol to same subjects as in Fig. 5. Shaded areas represent the steroids released by β-glucuronidase hydrolysis.

prolonged steroid therapy and the results compared to the mean of 5 normal subjects. The plasma levels of radioactivity readily extractable by chloroform (free steroids) and those of radioactivity released by β-glucuronidase hydrolysis (glucuronidates) are shown in Fig. 5. The rate of clearance of free steroids was essentially normal in Subject R. M. The much lower concentrations of "free" activity in Patient L. S. may have been due to severe, generalized edema present at the time in this patient. In Fig. 6 are shown urinary concentrations of radioactivity in these patients. None of the subjects excreted more than 3 per cent of the radioactivity administered in the free form.

2. *Adrenocortical Insufficiency*.—Standard ACTH tests of adrenocortical capacity were performed in a group of patients with known or suspected Addison's disease. In Table I are shown the 17 OHCS levels in the plasma of patients with proved Addison's disease during the administration of ACTH. The results are correlated with some of the clinical data. For complete interpretation of the results in this table it should be stressed that the plasma 17 OHCS were determined by the method of Silber and Porter[5] as modified by Eik Nes.[6] The underlined levels of 17 OHCS in Table I are from samples exhibiting a peak absorbancy at 410 mμ in the Porter Silber reaction[11] similar to the absorbance of 0.5 μg of crystalline cortisol taken through the entire procedure. This peak was not present in the remaining (not underlined) samples. The peaking at 410 mμ is highly indicative that the measured levels are due to the presence of minute quantities of steroids exhibiting a 17, 21 dihydroxy-20 ketone side chain (cortisol and some of its metabolites). The

TABLE I PLASMA 17 OHCS DURING 6 HOUR ACTH INFUSION IN SUBJECTS WITH ADRENAL INSUFFICIENCY

SUBJECT	AGE AND SEX	μG 17 OHCS/100 ML OF PLASMA DURING ACTH INFUSION				MAINTENANCE MEDICATIONS		DURATION OF DISEASE (YEARS)
		0 HR	2 HR	4 HR	6 HR	CORTISONE ACETATE MG./24 HR TAKEN ORALLY	DOC TMA*	
M	51 M	0.4	0.4	0.4	0.3	37.5	20	1
Ste	35 M	0	0	0	0	25	30	5
H	50 F	0.8	$\underline{0.9}$	$\underline{1.3}$	$\underline{2.0}$	25 37.5	25	13
Sto	45 F	0	0	0	0	37.5	30	3.4
Cf	30 F	0.9	$\underline{1.9}$	$\underline{2.1}$	$\underline{1.0}$	25	20	12
W	47 M	0	0	0	0	12.5	40	12
Sw	40 F	0.2	$\underline{2.9}$	$\underline{3.7}$	$\underline{3.2}$	37.5	40	5
P	33 M	0.3	1.0	0.9	0.7	25	40	16
B	19 F	0	0	0	0	37.5	50	6
N	53 F	0	0	0	0	25	62	16
Ch	19 M	1.7	1.6	$\underline{2.9}$	$\underline{5.2}$	25	25	1
R	64 M	4.0	1.3	1.8	1.7	37.5	30	1
Normals		$\underline{11}$	$\underline{27}$	$\underline{35}$	$\underline{40}$			

*Deoxycorticosterone trimethylacetate
For explanation of underlining see text

method used[6] for the determination of the 17-OHCS levels will not measure accurately concentrations of 17-OHCS below 1.2 µg/100 ml. of plasma. The range for normal subjects between 9 A.M. and 2 P.M. is 7 to 15 µg/100 ml. of plasma, the average being 10 µg.

From Table I it can be seen that none of the patients with Addison's disease had control values of plasma 17-OHCS within the normal range, and only 2 of the control values fell within the reliable range of the accuracy of the method. In 4 of the subjects the administration of ACTH resulted in the appearance in plasma of cortisol-like substances (underlined values) as indicated by their absorption at 410 mµ. None of the patients, however, showed a response to ACTH which approached that of normal subjects. There was no apparent correlation between the clinical picture of these patients and the appearance of cortisol in the plasma.

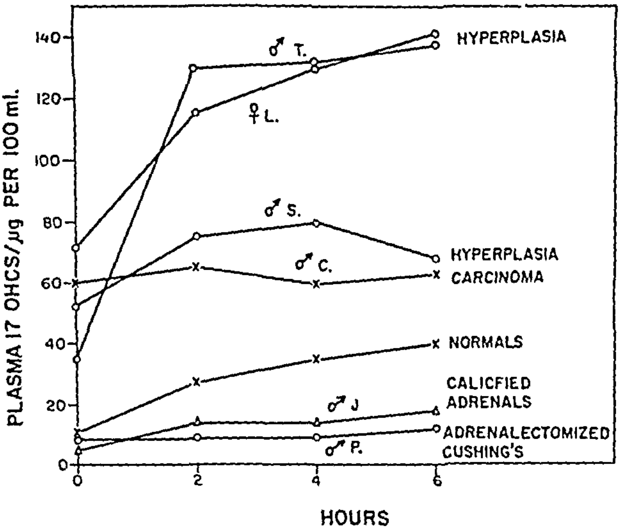

Fig. 7.—Plasma 17-OHCS levels in subjects with adrenal disease. Note lack of response of patient with carcinoma of the adrenal gland and exaggerated responses of patients with adrenal hyperplasia.

In Fig. 7 are shown the changes in the plasma 17-OHCS levels in a subject (J.) with calcification of both adrenal glands. This 30-year-old man had noticed definitely increased pigmentation of his skin and salt-craving. The response of the 17-OHCS to the administration of ACTH is definitely subnormal and may indicate partial adrenal insufficiency.

3. *Adrenal Hyperfunction.*—Changes in plasma 17-OHCS levels during the intravenous infusion of ACTH were determined in 4 patients with Cushing's syndrome. The results are shown in Fig. 7. In all the cases the control plasma 17-OHCS levels were repeatedly much higher (35 to 82 µg) than those seen in normal subjects (7 to 15 µg).

Two of the patients with adrenal hyperplasia (T. and L.) showed responses to the infusion of ACTH which were of much greater magnitude than those seen in normal subjects. The response of Patient S. with adrenal hyperplasia was not as prominent as that seen in Patients T. and L., and was similar to the response of normal subjects No significant changes in the plasma 17 OHCS levels occurred in the male subject with Cushing's syndrome due to carcinoma of the left adrenal gland with metastases to the lung Subject P was a man with Cushing's syndrome due to bilateral adrenal hyperplasia for which bilateral total adrenalectomy had been performed More than a year after the operation the plasma from this patient contained normal levels of 17 OHCS, which did not rise significantly when ACTH was administered On another occasion, when the patient was taking 0 5 mg of fluorohydrocortisone 4 times daily, and his urinary 17-OHCS ranged from 0 70 to 1 05 mg per day, the administration of 80 units of ACTH daily for 5 days resulted in a rise of the urinary 17 OHCS, with the first day's excretion being 1 84 mg and the fifth day's 4 23 mg These findings indicate that even though bilateral adrenalectomy had been performed on this patient some functioning adrenocortical tissue was still present in the patient, although it was sluggish in its response to ACTH

DISCUSSION

That the administration of adrenal steroids will cause atrophy and hypofunction of the adrenal cortex of animals has been known for many years [12] These findings have been corroborated in human subjects during the administration of therapeutic doses of cortisol or cortisone [13] [14] The results of the present study, based on plasma 17 OHCS levels, confirm the profound depression of adrenocortical capacity produced by long term steroid therapy but also indicate the considerable individual variation in the speed with which adrenocortical function may return to normal Examination of the experimental and clinical data fails to reveal any significant correlation between the dose of steroid taken, the length of time of steroid therapy, the depression of the plasma 17 OHCS levels, and the response of the plasma 17 OHCS to ACTH In one subject (L C) the response of the plasma 17 OHCS did not return completely to normal even after 4 successive daily intravenous administrations of ACTH, whereas Subject M G showed a fairly normal response during the first day Following gradual reduction and then discontinuation of cortisone therapy, Patient W N also had a normal elevation of 17 OHCS levels following the stress of an acute infection

The rates of metabolism of a 50 mg dose of cortisol in patients who had been on prolonged and intensive steroid therapy did not differ significantly from that observed in control subjects The metabolism of 4 C^{14}-cortisol was essentially normal in one of the two patients who received a tracer dose of the radioactive steroid The other subject (L S), at the time the radioactive cortisol was injected, had severe, generalized edema and a clinical picture compatible with the nephrotic syndrome complicating lupus erythematosus The lower levels of radioactivity in the plasma free steroid fraction may be related to migration of C^{14} cortisol into the large extravascular space Previous studies have shown that 17 OHCS may appear in extravascular fluids [15] Altered metabolism, however, of C^{14} cortisol cannot be ruled out in Patient L S

From the data presented it is apparent that most patients with definite clinical evidence of Addison's disease do not have significant amounts of 17-OHCS in the plasma, even after maximal stimulation with ACTH. In several patients, however, the administration of ACTH resulted in low but definite concentrations of 17-OHCS in plasma indicating the presence of small amounts of adrenocortical tissue. Most, but not all, patients with Addison's disease have been shown to have demonstrable amounts of ACTH in their blood, whereas this is not so for most normal subjects and for patients with Cushing's syndrome.[16-18] The presence of demonstrable ACTH activity in the bloods of most subjects with adrenal insufficiency has been postulated to be due to lack of circulating corticosteroids which regulate and inhibit ACTH production by the pituitary. In patients with Addison's disease who may have small amounts of functioning adrenocortical tissue one might expect maximum stimulation of the tissue by the high circulating levels of endogenous ACTH and hence no response of the plasma 17-OHCS levels to the exogenous ACTH. Nevertheless, in 4 of the patients of the present study definite, albeit subnormal, elevations in circulating 17-OHCS were seen during the ACTH infusions. It is possible that these subjects did not have elevated endogenous blood ACTH levels, and hence, the small remnants of adrenocortical tissue responded to the large amounts of exogenous ACTH. In this connection, it is interesting to note that Addison's disease due to unitropic (ACTH) deficiency of the pituitary gland has been reported recently.[19] It is possible that some of the subjects in the present study who responded partially to ACTH may suffer from such a deficiency.

The existence of partial adrenal insufficiency is indicated by the subnormal response to ACTH of some of the patients who had been on prolonged corticosteroid therapy and by Subject J. A similar picture of partial adrenocortical insufficiency has been described previously.[7, 20]

The changes in the plasma 17-OHCS of patients with Cushing's syndrome produced by the administration of ACTH indicate that in most cases of adrenocortical hyperplasia the response is exaggerated, occasionally to an extreme degree. On the other hand, in the patient with carcinoma of the adrenal no significant changes in the plasma 17-OHCS were apparent. A previously published report indicated that of 3 cases with Cushing's syndrome the subject with adrenocortical carcinoma exhibited an "inflexible, autonomous type of increased steroid production and excretion unaffected by ACTH."[21] On the other hand, the patient with an adrenocortical adenoma and the subject with adrenal hyperplasia exhibited a "flexible type of increased corticosteroid production and excretion which responded in an exaggerated manner to ACTH stimulation."[21] Recently, one of us[22] made a review of the cases of Cushing's syndrome followed in several clinics. The effect of ACTH administration (25 I.U. intravenously over 4 to 6 hours) on plasma and/or urinary 17-OHCS was studied in 56 cases of bilateral adrenal hyperplasia, 11 cases of unilateral benign adenoma, and 11 cases of adrenocortical carcinoma. In the hyperplasia group, 51 patients exhibited a hyperresponse, 4 a normal response, and 1 no significant change. Of the 11 cases of adenoma, 8 showed a hyperresponse, 1 a normal response, and 2 no change Finally, 2 patients with carcinoma of the adrenal cortex had a hyperresponse, 1 a normal response, and 8 did not respond

From the results presented it would appear that the determination of the plasma 17 OHCS levels may give us important information in the evaluation of adrenocortical function The capacity of the adrenal cortex is not an all-or none phenomenon, but one showing considerable variation from subject to subject and within one clinical entity, e g, adrenal insufficiency or hyperfunction

SUMMARY

Plasma 17 OHCS levels and adrenocortical capacity were greatly reduced in most patients on long term steroid (cortisol, cortisone) therapy In some subjects however, the adrenal cortex showed remarkable ability in returning to normal function after abrupt cessation of steroid therapy The rate of metabolism of intravenously administered cortisol was essentially normal in subjects following abrupt cessation of prolonged steroid therapy, although the plasma 17-OHCS concentrations tended to be higher in 2 of the patients Four of 12 patients with Addison's disease had low levels of 17 OHCS during ACTH infusion Two of 3 patients with Cushing's syndrome due to adrenocortical hyperplasia showed exaggerated plasma 17 OHCS levels during ACTH infusion, while the third patient had a fairly normal response A patient with functioning carcinoma of the adrenal showed elevated 17 OHCS levels with no changes during ACTH infusion

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LABORATORY METHODS

A COLORIMETRIC PROCEDURE FOR THE DETERMINATION OF SERUM GLUTAMIC OXALACETIC TRANSAMINASE

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THE possible clinical significance of an increased serum glutamic oxalacetic transaminase (GOT) level, was reported by La Due, Wroblewski, and Karmen.[4,8] Numerous publications have since appeared in which the transaminase activity has been employed as a diagnostic tool[7,9,13]; increased serum transaminase activity has been reported in pathologic conditions involving myocardial infarction,[1,6,8] hepatocellular damage,[1,13] obstructive jaundice,[1] and acute pancreatitis.[1]

Karmen[5] devised a spectrophotometric method for determining GOT activity. The assay is based on the transamination of aspartic acid with α-ketoglutaric acid to form oxalacetic acid and glutamic acid (Equation I); as oxalacetic acid is formed, it is reduced to malic acid in the presence of exogenous malic dehydrogenase and reduced diphosphopyridine nucleotide (DPNH*) (Equation II). The amount of DPNH* oxidized to DPN is determined with an ultraviolet spectrophotometer and expressed as transaminase units.

Equation I:

$$\underset{\text{Aspartic Acid}}{HOOC\,CH_2\,CHNH_2\,COOH} + \underset{\alpha\text{ Ketoglutaric Acid}}{HOOC\,CH_2\,CH_2\,CO\,COOH} \;\underset{\xleftarrow[\text{Phosphate Buffer pH 7.1}]{}}{\xrightarrow[37^\circ\text{ C.}]{\text{Transaminase}}}$$

$$\underset{\text{Oxalacetic Acid}}{HOOC\,CH_2CO\,COOH} + \underset{\text{Glutamic Acid}}{HOOC\,CH_2\,CH_2\,CHNH_2\,COOH}$$

Equation II

$$\underset{\text{Oxalacetic Acid}}{HOOC\,CH_2CO\,COOH} + \underset{\text{Co. I. }H_2}{DPNH} + \underset{\xleftarrow{\phantom{\text{Malic Dehydrogenase}}}}{\xrightarrow[\text{Phosphate Buffer}]{\text{Malic Dehydrogenase}}} \underset{\text{Malic Acid}}{HOOC\,CH_2CHOH\,COOH} + \underset{\text{Co. I}}{DPN}$$

Equation III

$$\underset{\text{Oxalacetic Acid}}{HOOC\,CH_2\,COOH} \xrightarrow[37^\circ\text{ C}]{\text{Aniline Citrate}} \underset{\text{Pyruvic Acid}}{CH_3\,CO\,COOH} + CO_2$$

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The method of assay described in this paper does not involve the use of an ultraviolet spectrophotometer or DPN. It is a modification of the procedure described by Tonhazy, White, and Umbreit[12] for the determination of transaminase activity in tissue.* The assay is similar to the Karmen method in that the same basic transamination reaction (Equation I) is involved. However, the reduction of oxalacetic acid to malic acid (Equation II) is replaced by the conversion of oxalacetic acid to pyruvic acid by aniline-citrate (Equation III), and a subsequent quantitative determination of pyruvic acid by the formation of its 2,4-dinitrophenylhydrazine derivative in a manner similar to that described by Friedemann and Haugen.[2]

REAGENTS AND EQUIPMENT

1. Buffer, pH 7.4 (0.1 M): Sodium Phosphate Buffer (2.30 Gm. $Na_2HPO_4 \cdot H_2O$ and 11.83 Gm. NaH_2PO_4 [anhydrous] dissolved in water and diluted to one liter).

2. Sodium Aspartate (0.2 M): 0.665 Gm. DL-aspartic acid dissolved in 20 ml. of above buffer, pH adjusted to 7.4 with 2.5 N KOH and diluted with buffer to 25 ml. Small portions of this and the following solution were placed in tubes and stored at -20° C.

3. Sodium α-ketoglutarate (0.1 M): 0.365 Gm. α-ketoglutaric acid dissolved in 20 ml. of buffer pH adjusted to 7.4, with 2.5 N KOH and with buffer diluted to 25 ml.

4. Ten per cent metaphosphoric acid: 10 Gm. metaphosphoric acid dissolved in water and diluted to 100 ml.

5. Aniline-citrate: 100 Gm. citric acid dissolved in water and diluted to 100 ml. Immediately prior to use equal amounts of C.P. aniline, which was not redistilled, and citric acid were mixed together.

6. 2,4-dinitrophenylhydrazine reagent (0.1 per cent): 0.1 Gm. 2,4-dinitrophenylhydrazine dissolved in 20 ml. concentrated (37 per cent), HCl, diluted to 100 ml. with water.

7. Water saturated toluene: 100 ml. of reagent grade toluene shaken thoroughly with 10 to 15 ml. of water in a separatory funnel. The excess water is removed, and the toluene is stored in a brown bottle, at 4° C.

8. Alcoholic KOH (2.5 per cent): 2.5 Gm. KOH dissolved and diluted to 100 ml. with 95 per cent ethanol. This was prepared daily.

9. Sodium Pyruvate Standard: Sodium pyruvate was prepared according to Robertson.[10] Ten milliliters of redistilled pyruvic acid, dissolved in 100 ml. ethanol, was neutralized with alcoholic NaOH (10 ml. water saturated NaOH diluted with 100 ml. ethanol) with constant stirring in an ice bath. Sodium pyruvate precipitated out as a white amorphous powder. This precipitate was collected on a Buchner funnel and washed four times with 50 ml. of cold ethanol, and twice with 25 ml. of cold ethyl ether. The white powder was dried over calcium chloride for 18 hours in a vacuum desiccator. A stock solution containing 800 mcg. of pyruvic acid per milliliter was prepared by dissolving 100 mg. of sodium pyruvate in 100 ml. 0.1 M PO_4 buffer pH 7.4.

10. Photoelectric Colorimeter with 450 mμ filter.

PROCEDURE

The enzyme assay was performed in the following manner: To 2 (18 × 150 mm.) test tubes, the following were added:

TEST	*BLANK* (Zero Time Control)
0.2 ml. serum	0.2 ml. serum
0.3 ml. buffer	0.3 ml. buffer
--------------	0.3 ml. 10 per cent metaphosphoric acid
0.5 ml. DL-aspartate	0.5 ml. DL-aspartate

---

*Following the development of this method, we were informed that Dr. W. W. Umbreit adapted the Tonhazy, White, and Umbreit procedure for the determination of serum glutamic oxalacetic transaminase.

	Mix and incubate for 30 minutes at 37° C	
0.2 ml α ketoglutarate		0.2 ml α ketoglutarate
	Mix and incubate for 10 minutes at 37° C	
0.3 ml 10 per cent metaphosphoric acid		---- -- -- - - --

Two drops of aniline citrate are added to each tube, and incubated for 10 minutes at 37° C. One milliliter of the 0.1 per cent 2,4 dinitrophenylhydrazine reagent is then added to each tube, and the tubes again incubated for 5 minutes at 37° C. The tubes are removed from the water bath and 3.0 ml. of water saturated toluene is added, to extract the pyruvo phenylhydrazine. The tubes and their contents are mixed vigorously, and then centrifuged for 5 minutes. The toluene layer is removed with a capillary pipette, and placed in a separate tube. One milliliter of the toluene extract is placed in a colorimeter tube containing 5 ml. of 2.5 per cent alcoholic KOH solution and 0.5 ml. of water. The contents in the tube are mixed and allowed to stand at room temperature for 10 minutes. The tubes are read at 450 mμ in a Bausch and Lomb "Spectronic 20" photoelectric colorimeter. The colorimeter was zeroed (blanked out) with the zero time control and the optical density obtained with the test was read against a standard pyruvic acid curve to determine the amount of pyruvic acid. A standard control, containing 100 mcg. of sodium pyruvate is incorporated in each series of determinations.

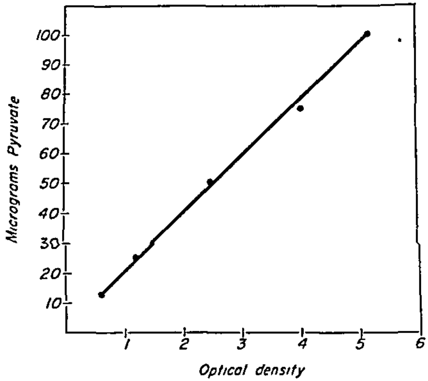

Fig. 1.—Relationship of sodium pyruvo dinitrophenylhydrazine to optical density.

STANDARDIZATION

This method was standardized by the addition of varying amounts of sodium pyruvate, in 0.3 ml. quantities, to a zero time control in lieu of 0.3 ml. of buffer, and treated in a similar manner as the zero time control system. The standard was read against a zero time control, and a straight line relationship was found to exist between the concentration of sodium pyruvate and optical density. (See Fig. 1.)

CALCULATIONS

A Karmen unit is defined as the enzymatic activity of 1 ml. of serum which would oxidize DPNH* (Equation II) resulting in an optical density change of 0.001 at 340 mμ in 1 minute. The Karmen procedure requires 200 mcg. of DPNH⁺ in a 3.0 ml. system, or 66.66 mcg. per milliliter. The micromolar extinction coefficient of DPNH⁺ being 6.22/ml.,[3] one micromol in 3 ml. would give an optical density value of 6.22 or 2.07/ml.

A serum containing, say, 500 Karmen units per milliliter would produce an optical density change of 5.000 in 10 minutes. This is equivalent to the formation of (5.000/2.07) 2.42 μM of pyruvate or (2.42 × 88) 213 mcg. of pyruvate.

The outlined procedure requires 0.2 ml. of serum in a 1.5 ml. reaction system, which is equivalent to 0.4 ml. of serum in a 3.0 ml. reaction system. Four tenths milliliter of this serum, containing 500 Karmen units, would, therefore, produce (0.4 × 214) 85.2 mcg. of pyruvate. Consequently 0.2 ml. of this serum in a 1.5 ml. reaction system would produce 42.1 mcg. of pyruvate.

The following equation has been employed to convert the obtained optical density values to Karmen units:

$$\frac{\text{O. D. of Test}}{\text{O. D. of 42.1 mcg. Pyruvate}} \times 500 = \text{Karmen units.}$$

RESULTS AND DISCUSSION

The validity and specificity of the Tonhazy, White, and Umbreit method for the determination of transaminase activity in serum was verified by employing complete, incomplete, and additive systems. Table I depicts the results obtained with such systems.

TABLE I. OPTICAL DENSITY VALUES OF PYRUVO-DINITROPHENOLHYDRAZONE OBTAINED WITH COMPLETE, INCOMPLETE, AND ADDITIVE SYSTEMS

SYSTEM*	OPTICAL DENSITY†	OPTICAL DENSITY‡
Inactivated serum§	.620	—
Serum + aspartate	.678	.056
Serum + α-ketoglutarate	.638	.022
Complete system	.796	.174
Complete system + 250 μg pyruvate	.905	.284
Complete system + 250 μg oxalacetate	.900	.278

*Reaction terminated with 0.3 ml. 100 per cent TCA.
†Colorimeter zeroed (blanked out) with water.
‡Colorimeter zeroed (blanked out) with inactivated serum.
§Serum inactivated with 0.3 ml. 100 per cent TCA.

Increasing the incubation time and/or the amount of serum yielded a proportional increase in the amount of pyruvo-dinitrophenylhydrazine. These data are presented in Table II. The addition of pyridoxal phosphate was without effect.

TABLE II. EFFECTS OF INCUBATION TIME AND VARIOUS AMOUNTS OF SERUM ON THE FORMATION OF PYRUVO-DINITROPHENOLHYDRAZONE

INCUBATION TIME (MIN.)	ML. OF SERUM	ML. OF BUFFER	PYRUVO-DINITROPHENOLHYDRAZONE (MCG.)
0.0	0.2	0.0	0.0
2.5	0.2	0.0	26.4
5.0	0.2	0.0	51.0
7.5	0.2	0.0	75.8
10.0	0.2	0.0	102.8
10.0	0.0	0.2	0.0
10.0	0.05	0.15	25.6
10.0	0.10	0.10	48.2
10.0	0.15	0.05	70.7
10.0	0.20	0.00	104.6

TABLE III. COMPARISON OF ZERO TIME CONTROL AND TESTS WITH DIFFERENT CONCENTRATIONS OF METAPHOSPHORIC ACID AND TRICHLOROACETIC ACID

SYSTEM	ACID—0.3 ML. (PER CENT)	OPTICAL DENSITY
Zero time control	100 Metaphosphoric	0.183
Test	100 Metaphosphoric	0.387
Zero time control	100 Trichloroacetic	1.398
Test	100 Trichloroacetic	1.699
Zero time control	10 Metaphosphoric	0.147
Test	10 Metaphosphoric	0.347
Zero time control	10 Trichloroacetic	0.830
Test	10 Trichloroacetic	1.032

TABLE IV. COMPARISON OF TRANSAMINASE ACTIVITY VALUES* OBTAINED WITH THE KARMEN METHOD, TONHAZY, WHITE, AND UMBREIT METHOD (TCA) AND OUR MODIFICATION (METAPHOSPHORIC ACID)

SERUM NO.	KARMEN†	TCA	METAPHOSPHORIC	DIAGNOSIS
1	0,2	11,15	0,0	Normal
2	[illegible]	11,20	5,8	Normal
3	7,9	15,20	10,13	Normal
4	25,28	40,48	27,33	Normal
5	[illegible]8,40	56,63	40,41	Normal
6	40,42	60,75	41,45	Normal
7	5[illegible],55	70,82	54,58	Normal
8	57,59	71,80	60,65	Normal
9	60,63	75,94	60,68	Normal
10	70,74	9[illegible],105	75,80	Normal
11	55,57	80,93	59,62	Cirrhosis of liver
12	105,112	140,173	111,117	Cirrhosis of liver
13	[illegible]5,[illegible]8	61,78	[illegible]9,4[illegible]	Cirrhosis of liver
14	450,460	[illegible]20,380	470,485	Acute hepatitis
15	790,805	530,610	819,840	Acute hepatitis
16	825	590	865	Chronic hepatitis
17	1090	635	1135	Chronic hepatitis
18	168	206	175	Hepatitis
19	605	425	638	Acute myocardial infarction
20	310	400	335	Acute myocardial infarction
21	184	250	196	Myocardial infarction
22	62	94	66	Myocardial infarction
23	73	100	77	Myocardial infarction
24	148	183	155	Myocardial infarction
25	130	174	136	Myocardial infarction
26	100	130	105	Myocardial infarction
27	191,197	242,278	20[illegible],209	Constrictive pericarditis (etiology, tuberculosis)
28	349	410	367	Rheumatic heart disease
29	236	276	241	Thorazine hepatitis
30	68	87	71	Pulmonary embolism with pleural effusion
31	15	31	19	Obstructing ulcer
32	93	150	100	Carcinomatosis
33	144,147	186,210	148,153	CA of ascending colon with hepatic metastasis
34	88,92	148,182	93,100	Grand mal epilepsy
35	150	193	155	Obstructive jaundice
36	18	48	22	Pneumonia
37	3	17	5	Pneumonia
38	60	88	64	Epilepsy
39	20	28	20	Multiple sclerosis
40	80	125	84	Bronchogenic carcinoma
41	160,165	215,258	168,175	Acute leukemia
42	27	52	31	Cerebral thrombosis

*All values are expressed as Karmen units.

†Preincubation period as well as the 10 minute photometric analysis were performed at 37° C.

When the original method was checked against the Karmen procedure (performed at 37° C.) much larger and poorly reproducible values were obtained. This we believe was due to the intense chromogen formed with the 100 per cent trichloroacetic acid (TCA) (Table III). To compensate for this, 10 per cent metaphosphoric acid or 10 per cent TCA was used in lieu of the 100 per cent TCA and a 30-minute preincubation period with aspartic acid was instituted. Both of these modifications yielded results approximately 10 per cent higher than those obtained with the original Karmen procedure, as well as a reproducibility factor of approximately 5 per cent. Table IV depicts the transaminase activities of sera compared by the Karmen method, the Umbreit method, and our modification.

SUMMARY

A colorimetric procedure for the determination of serum glutamic oxalacetic transaminase, which does not require either an ultraviolet spectrophotometer or reduced DPN, is described. The procedure is a modification of that which has been employed by Tonhazy, White, and Umbreit. The following modifications were found necessary to insure a reproducible correlation between the amounts of sodium pyruvo-dinitrophenylhydrazine, and transaminase units as defined by Karmen:

1. The serum was preincubated with the aspartic acid for 30 minutes.
2. Ten per cent metaphosphoric acid or 10 per cent TCA, rather than 100 per cent trichloroacetic acid was employed to terminate the reaction.

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VARIATION IN COMPLEMENT FIXATION ANTIGENICITY OF DIFFERENT YEAST PHASE STRAINS OF HISTOPLASMA CAPSULATUM

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INTRODUCTION

IN A recent study Schubert and his associates[1] demonstrated that strains of *Histoplasma capsulatum* differed in their ability to produce Histoplasmin of satisfactory antigenicity. The objective of the present investigation was to determine whether variations in potency occurred in a group of antigens derived from strains of *H. capsulatum* in the yeast phase.

MATERIALS AND RECORDS

Eleven strains of *H. capsulatum* were converted to the yeast phase by means of serial subcultures on cotton-plugged tubes of blood-free brain heart infusion agar* incubated at 37° C. Four-day-old yeast phase growths were harvested in sterile physiologic saline containing 0.01 per cent Merthiolate. After storage at room temperature for 48 hours, the suspensions were filtered through gauze, centrifuged, and the cells resuspended in Merthiolate-saline solution. The antigens then were adjusted volumetrically to produce a 10 per cent stock suspension of cells in barbital-buffered saline. Subsequently each yeast phase antigen was tested for anticomplementary activity as well as antigenicity by means of a box titration. The optimum dilution of antigen for each strain was selected on the basis of agreement noted in box titrations on several human sera from proved cases of histoplasmosis. Tests on rabbit sera were performed with optimum antigen dilutions as determined by box titrations with the homologous rabbit antisera.

The strains of *H. capsulatum* employed in this study and their sources are cited in Table I. All of the strains were recovered from human cases of histoplasmosis except for strain B11, which was isolated from a soil sample collected in a Peruvian cave by Lazarus and Ajello.[3]

Sera.—(1) Hyperimmune rabbit sera were prepared by injecting rabbits with suspensions of the yeast phase strains prepared in physiologic saline containing 0.3 per cent formalin and adjusted to a McFarland No. 3 density. Intravenous injections of 0.5, 1.0, 2.0, and 3.0 ml. of the yeast phase suspension were made at 4-day intervals. The rabbits were bled 7 days after the last injection.

(2) Human sera were obtained from culturally proved cases of histoplasmosis and from persons presumably infected by *H. capsulatum* as indicated by pulmonary lesions, clinical symptoms, and a positive Histoplasmin skin test. Sera were tested in serial dilutions beginning with a dilution of 1:8.

Complement Fixation Procedure.—The complement fixation procedure used was an adaptation of the 50 per cent end point technique, employing barbital-buffered physiologic saline throughout (Mayer et al.[2]). Antigens and serial dilutions of inactivated sera were

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Note: This is not an endorsement by the Public Health Service but rather a means of identifying a particular product.

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tested in 0.25 ml. of complement containing 5 fifty per cent units. Fixation was allowed to proceed for 15 to 18 hours at 4 to 6° C. The tubes then were placed at room temperature while the sensitized cells (a 2 per cent suspension of sheep erythrocytes sensitized for 10 minutes in the proportion of 1:1 with 4 units of hemolysin in 0.25 ml.) were prepared. The sensitized cells were added in 0.5 ml. amounts and the tests read after a secondary incubation period of 30 minutes in a 37° C. water bath. The titers were recorded as the highest dilutions of serum which gave a 3 plus or 4 plus reaction (30 per cent hemolysis or less).

TABLE I. SOURCE OF HISTOPLASMA CAPSULATUM CULTURES CONVERTED TO YEAST PHASE

STRAIN	SOURCE
CDC* Number	
28	N. F. Conant—1066, Duke University Medical School
105	N. F. Conant—1071, Duke University Medical School
267	Communicable Disease Center Collection
A228	M. L. Furcolow, Communicable Disease Center
A232	M. L. Furcolow, Communicable Disease Center
A233	M. L. Furcolow, Communicable Disease Center
A235	M. L. Furcolow, Communicable Disease Center
A811	C. W. Emmons—F6624, National Institutes of Health
A827	C. C. Campbell—G2, Army Medical School
B11	Communicable Disease Center Collection, Peruvian soil isolate
B12	S. B. Salvin—6515, National Institutes of Health

*Communicable Disease Center

The complement binding power of the antigen was tested with 1.25, 2.5, and 5 fifty per cent units of complement. The antigen was considered to be anticomplementary when less than 80 per cent hemolysis was obtained with 2.5 fifty per cent units.

RESULTS

The comparative antigenicity of each yeast phase strain was tested first with the sera from immunized rabbits in order to determine whether the degree of reactivity obtained with rabbits could be used as a guide for selecting an antigen for the testing of human sera. The results obtained with human and rabbit sera are presented in Tables II and III. The results demonstrated that the 11 different strains of *H. capsulatum* developed antigens which showed wide ranges of activity, especially in tests with human sera (Table III).

TABLE II. COMPLEMENT FIXATION TITERS OBTAINED WITH ELEVEN STRAINS OF YEAST PHASE HISTOPLASMA CAPSULATUM AGAINST HOMOLOGOUS AND HETEROLOGOUS RABBIT ANTISERA

ANTIGENS		RABBIT SERUM ANTIBODY TITERS									
STRAIN	DILUTION*	28	105	A228	A232	A235	267	A811	B11	B12	A827
28	1:32	*2,018*	128	1,024	1,024	256	1,024	4,096	64	128	2,048
105	1:8	256	*32*	128	256	64	256	512	32	64	256
A228	1:32	1,024	64	*512*	1,024	256	512	4,096	32	128	1,024
A232	1:32	512	32	256	*512*	128	512	1,024	16	128	1,024
A233†	1:32	512	32	256	512	128	512	512	32	128	1,024
A235	1:32	1,024	64	512	1,024	*256*	512	1,024	32	256	2,018
267	1:32	512	16	256	256	128	*4,096*	1,024	16	64	2,048
A811	1:64	1,024	64	512	1,024	128	4,096	*1,024*	32	256	1,024
B11	1:64	64	0‡	128	256	64	256	512	*128*	32	128
B12	1:64	1,024	32	256	512	128	1,024	512	16	*128*	1,024
A827	1:16	512	64	256	512	256	512	1,024	32	256	*4,096*

Homologous antiserum shown by italics.
*Determined by box titrations with homologous sera.
†233 serum insufficient.
‡Less than 1:8.

In an attempt to grade the strains in respect to their antigenic activity, the data in Tables II and III were rearranged in the order of the median titers obtained with the human and rabbit sera tested. The results, presented in Table IV, show that the first 6 strains listed (28, A811, A228, A235, A827, and 232) gave higher median titers than the other 5 antigens and the orders of their listings were the same with both types of sera. Strain 28 gave the highest median titers and strain A811 gave the next highest titers with both groups of sera. The last two strains listed (105 and B11) were definitely inferior antigenically, giving the lowest median titers with both human and rabbit sera. The remaining three strains, 267, A233, and B12 were intermediary in position, giving definitely higher median titers than B11 and 105 when tested with human and rabbit sera.

TABLE III. COMPLEMENT FIXATION TITERS OBTAINED ON HUMAN SERA FROM CASES OF HISTOPLASMOSIS WITH 11 YEAST-PHASE HISTOPLASMA CAPSULATUM ANTIGENS

ANTIGENS		HUMAN SERUM ANTIBODY TITERS									
STRAIN	DILUTION*	1†	2	3	4	5	6‡	7	8	9	10
28	1:32	1,024	64	1,024	128	2,048	256	4,096	256	1,024	128
105	1:8	8	0§	0	8	32	16	16	0	32	8
A228	1:32	512	32	512	64	1,024	128	1,024	64	512	64
A232	1:32	512	32	512	64	512	64	512	128	512	64
A233	1:32	512	32	512	0	512	64	1,024	128	128	0
A235	1:32	512	16	512	64	1,024	128	1,024	128	512	64
267	1:32	512	16	512	128	512	128	2,048	128	512	128
A811	1:64	512	32	1,024	32	1,024	256	1,024	256	512	32
B11	1:64	0	0	0	0	8	16	16	0	0	0
B12	1:64	256	16	512	32	256	32	128	64	256	32
A827	1:16	512	32	512	64	512	64	512	128	512	64

*Determined by box titrations.

†Sera 1 to 5 from culturally proved cases of histoplasmosis.

‡Sera 6 to 10 from presumptive cases of histoplasmosis.

§Titers less than 1:8.

TABLE IV. ANTIGENICITY OF ELEVEN STRAINS ARRANGED IN ORDER OF THE MEDIAN TITERS OBTAINED WITH TEN HUMAN AND TEN RABBIT SERA

HUMAN SERA		RABBIT SERA	
STRAIN	MEDIAN TITER	STRAIN	MEDIAN TITER
28	512	28	1,024
A811	256-512	A811	512-1,024
A228	256	A228	512
A235	256	A235	512
A827	256	A827	256-512
A232	256	A232	256-512
267	256	A233	256-512
A233	128	B12	256-512
B12	64-128	267	256
105	8	105	128-256
B11	0	B11	128

DISCUSSION

As in the study with Histoplasmins, Schubert et al.,[1] the results of this investigation indicate that yeast phase suspensions of various strains of *H. capsulatum* vary in their antigenic potencies, which illustrates the necessity of screening a large number of strains in order to select antigens of the highest reactivity for use in serologic tests.

A comparison of the [illegible] obtained with homologous and heterologous antisera (Table II) showed that the highest titers were not necessarily produced with homologous antigens

It is interesting to note that the results obtained in the box titrations of rabbit sera led to the use of the same optimum dilutions as had been determined by box titrations with human sera Such an observation is contrary to the experience obtained with Histoplasmin antigens in which the optimum antigen dilutions generally are higher with hyperimmunized rabbit sera

The data point out that [illegible] results obtained in various laboratories will be influenced by the particular strain from which the antigen is prepared In a previous study designed to compare the antigenicities of a yeast phase and a Histoplasmin antigen prepared from a single strain, Schubert and his associates[4] selected strain 105 because it had proved satisfactory as a producer of a good Hisoplasmin antigen for use in complement fixation tests and because it could be converted readily into the yeast phase The data presented here show that strain 105 produces a relatively poor yeast phase antigen which may account for the low yeast phase titers reported in the previous study

SUMMARY

The antigenicities of yeast phase suspensions derived from strains of *Histoplasma capsulatum* were determined through titrations of hyperimmunized rabbit sera and of human sera from culturally proved and presumptive cases of histoplasmosis Considerable variations in reactivities were noted and the results obtained were similar with human or rabbit sera Hyperimmune rabbit sera therefore may be used as control sera in the determination of the optimum yeast phase antigen dilutions To assure the selection of potent yeast phase antigens for use in complement fixation tests a large series of antigens derived from different strains of *H capsulatum* should be screened

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SEPARATION OF BONE SODIUM, POTASSIUM, AND CALCIUM BY THE USE OF CATION EXCHANGE RESIN

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INTRODUCTION

During the last ten years an increasing interest in the determination of the concentration of sodium and potassium in bone has arisen, due to the recognition of the possibly important role of the bone system in the sodium and potassium metabolism of the body.[3, 10, 12] Evidence in support of this has been presented by many investigators.[2, 3, 5, 10, 11, 13]

The main difficulty in such determinations in cortical bone has been the extremely high concentration of calcium in relation to the concentrations of sodium and potassium. The relationship of calcium:sodium:potassium is approximately 200:4:1 when calcium is expressed as milligrams of the secondary phosphate and the other two as their chlorides.

The methods published up to 1955 for the analysis of sodium and potassium in bone can conveniently be divided into the following three groups. (1) Wet ashing in concentrated nitric acid, or dry ashing followed by solution of the ash in hydrochloric acid and repeated precipitations of the calcium in the bone sample as carbonate or oxalate. The sodium and potassium content is determined in the supernatant by either flame photometry or chemical methods (e.g., gravimetric zinc uranyl acetate). (2) Primary determination of the calcium content of the bone sample followed by flame photometry of a second aliquot with a comparable amount of calcium introduced as a blank in the standards. (3) Solution of the bone with immediate flame photometry of the mixture, disregarding the calcium content. Some investigators have found these methods satisfactory.[3, 11, 14] Others have found them unsatisfactory,[4, 6, 9] the main problems have been interference by the calcium in the flame photometry of the sodium, and large coprecipitation of sodium when calcium is precipitated before sodium determination is done.

In our hands the above methods were unsuitable when applied to artificial mixtures with known content of secondary calcium phosphate, sodium chloride, and potassium chloride in the proportions found in cortical bone. With the precipitation technique the recovery was only 85 per cent and too variable, method two was found to be an elaborate procedure, and method three gave falsely high values. Separation of sodium from potassium in the bone sample was not possible by any of these techniques. We then proceeded to develop the method described in this paper. During this time the publications of Forbes and D'Ambrúsio[7] and Forbes and Lewis[8] appeared. They separated

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cations in bone with quantitative recovery of sodium by column chromatography, using a cation exchange resin (Dowex 50 × 12, 50 to 100 mesh). The bone samples were dissolved in 3 N HCl after preliminary drying and ashing in a muffle furnace.

Our method has many features in common with the method of Forbes, and for certain purposes it has advantages. The column used is smaller, and smaller bone samples can be examined with the same degree of accuracy owing to smaller volumes of eluate. It gives quantitative recovery of potassium in a separate fraction as well as of sodium. Preliminary drying and ashing of the bone is not necessary and this saves time and loss of material which is of importance when short-lived isotopes of sodium and potassium are employed (Na^{24} can be used instead of Na^{22}).[8]

MATERIALS AND METHODS

The cation exchange resin, Dowex 50, –400 mesh size, 12 per cent cross linked,* is made up in a column in a Pyrex glass tube of 12 mm. internal diameter and 20 cm. length. The lower end of the glass tube is melted to a conical shape with a narrow opening. In this cone some glass fragments form the base for a layer of Pyrex glass wool, acting as support for the resin. On top of the resin there is a layer of loosely packed glass wool which assures an even and gradual flow into the resin. The eluting fluid which is to pass through the column is held in a 500 ml. container with an opening in the bottom suitable for the attachment of thin polyethylene tubing. The free end of this tubing is attached to a thin Pyrex tube which is placed through an airtight rubber stopper on top of the column tube. All parts are supported by suitable clamps to a 4 foot stand (Fig. 1).

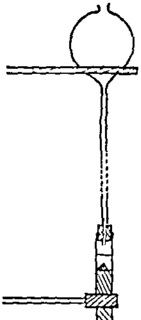

Fig. 1.—Diagram of column and container as described in text.

An air space between the rubber stopper and the fluid above the resin permits the rate of flow to be controlled with sufficient accuracy by counting the drops that pass per unit time. Regulation of the rate of flow is easily undertaken by moving the 500 ml. container up or down, thereby avoiding the use of complicated equipment for this purpose.[1] The reservoir is large enough to secure a constant rate of flow during the separation period. Cessation of flow is obtained by a clamp on the polyethylene tubing.

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*From Dow Chemical of Canada, Ltd., Montreal, Quebec.

The resin is immersed in distilled water, poured into the glass tube and allowed to settle to the required height. The height of the column will vary with different batches of resin, making it necessary to determine the elution curve of each batch (variation in height of column is from 10 to 15 cm.). The two elution curves shown (Figs. 2 and 3) are from two different batches. The resin is freed from unwanted cations by washing with 300 ml. of 3 N hydrochloric acid at a speed of 2 ml. per minute. Excess hydrogen ions are then removed by 200 ml. of distilled water. The column is then ready for use. A piece of bone of approximately 0.2 grams wet weight is the most satisfactory amount for a column of the size described. It should be freed from spongy bone, periosteum, and marrow. After weighing it is dissolved in 1 ml. of concentrated nitric acid by heating in a Pyrex test tube. When clear fluid is obtained the volume is made up to 10 ml. with distilled water and poured into the container, and an additional 10 ml. of water is used for washing of the test tube and added to the container. The 20 ml. of solution should fill the polyethylene tubing from the rubber stopper and up, before flow is started. It is also advisable to maintain a constant volume of distilled water on top of the resin prior to beginning each separation thereby avoiding changes in elution curves associated with variation in amount of fluid above the column. Normal hydrochloric acid is used as the eluting agent, with a rate of flow of 0.8 ml. per minute. The whole separation takes from 3½ to 4 hours. The sodium and potassium content of the eluate is determined in a Baird flame photometer.

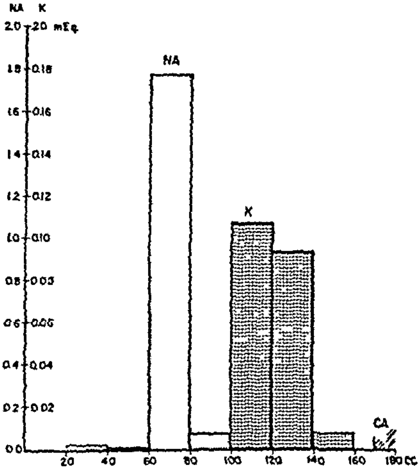

Fig. 2.—Elution curve for collection of sodium and potassium in same fraction (see text).

RESULTS

In Figs. 2 and 3 are shown two elution curves from bone biopsies. The first is useful when the removal of calcium is the only objective; the second shows the possibility of separating sodium and potassium in the same step as the removal of calcium. The volumes of eluate as it comes from the lower end of the column is plotted along the abscissa. The concentration of sodium and potassium in 10 c.c. samples are given in the ordinate. The potassium concentration is plotted in 10 times the scale of the sodium concentration, owing to the low concentration of the former. This gives a false impression of a significant overlap with potassium in the sodium fraction in Fig. 3. The actual content of potassium in the sodium fraction is only 0.00016 meq. The shaded area following the potassium peak is the spot where the first traces of calcium occur as detected qualitatively with solutions of ammonium carbonate and oxalate. The interspaces between the fractions are sufficiently large to make the separation a workable procedure. The reproducibility of the pattern is, however, dependent on a high degree of standardization with the least possible variation in the different steps in the preparation and working of the columns. Rather than washing the calcium out of the resin with large amounts of acid for renewed use, we have chosen to use fresh resin for each separation.

During the actual use of the columns the eluate is not collected in 10 ml samples but the sodium and potassium fractions are taken out in single volumes. In the elution curve in Fig. 2, sodium and potassium is collected from 30 to 140 ml. In Fig. 3 the collection of sodium is from 30 to 90 ml, potassium from 100 to 160 ml.

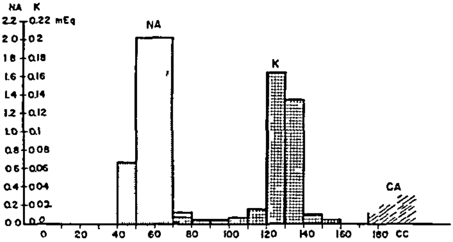

Fig. 3.—Elution curve for collection of sodium and potassium in separate fractions (see text).

The recovery rate in 6 consecutive experiments from artificial mixtures of secondary calcium phosphate, sodium chloride, and potassium chloride, with known amounts of cations simulating a 0.2 gram bone sample, was as follows:

(*a*) Sodium and potassium in one sample

Content in sample	*Recovery*	*Deviation from true content*
Sodium 0.05 meq	0.04913 ± 0.00055 meq	Less than 3 per cent
Potassium 0.016 meq	0.01511 ± 0.00017 meq	Less than 6 per cent

(*b*) Sodium and potassium in separate samples

Content in sample	*Recovery*	*Deviation from true content*
Sodium 0.05 meq	0.050533 ± 0.00018 meq	Less than 2 per cent
Potassium 0.01 meq	0.00938 ± 0.000074 meq	Less than 7 per cent

Attention should be paid to the extremely small standard deviations. The recovery of potassium is between 93 and 95 per cent, of sodium between 98 and 102 per cent.

DISCUSSION

In this laboratory more than 300 examinations of bone biopsies have been performed with the described technique. To date, none of the results indicates faulty separation or other technical difficulties. In most of the work radioactive sodium and potassium have been employed. Under these circumstances contamination with sodium in the potassium fraction would easily be detected

One of the early difficulties with the resin was its impermeability to water when made up in a column. This was caused by a variation in size of the resin particles. A more homogeneous size of the particles is obtained if the resin is immersed in distilled water and allowed to settle by standing. The lighter and smaller particles will then parcipitate last and can be poured off. Repetition of this procedure a few times will secure an easily permeable resin column. A slight contamination with potassium may be encountered even in analytically pure hydrochloric acid. The amounts present are usually too small to give any significant error in the potassium determinations, but a periodic check of the acid is justified.

SUMMARY

A technically simple method is described for the separation of bone sodium, potassium, and calcium by the use of a cation exchange resin in a column. The recovery of sodium is between 98 and 102 per cent, of potassium between 93 and 95 per cent.

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THE CHLAMYDOSPORES OF CANDIDA ALBICANS: COMPARISON OF THREE MEDIA FOR THEIR INDUCTION

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Candida albicans, the most common etiologic agent of moniliasis, can be identified readily if cultures produce submerged mycelium bearing spherical clusters of blastospores and characteristic chlamydospores. Production of chlamydospores is favored by a reduced oxygen tension, limiting nutrients, and a temperature of 18° to 25° C. They have the following morphology: (1) generally spherical, exceptionally oval, lacrimoid, pyriform or cylindrical, (2) enlarged in comparison with hyphae and blastospores, ranging in diameter from 6 to 17 μ, mostly 8 to 9 μ, (3) wall thick, hyaline, "double contoured", (4) borne terminally, but may be lateral or intercalary, when intercalary, usually cylindrical, (5) sometimes sessile, but usually pedunculated and borne on a distinctive subterminal cell, the "chlamydospore", (6) single or in short chains or clusters, (7) cytoplasm with one or more coarse, refringent droplets.

Since a limited supply of nutrients is essential for chlamydospore production, these structures will not generally be formed on the peptone carbohydrate media, such as Sabouraud's glucose agar, employed for the isolation of *C. albicans* from clinical specimens.

For induction of chlamydospores Benham recommended, in 1931, a corn meal infusion agar, a medium prepared by extracting 4 per cent corn meal in water at 60° C. for one hour, followed by filtration, reconstitution to original volume, and the addition of 1.5 per cent agar.

This corn meal infusion agar became widely adopted by many laboratories. The difficulties involved in preparing reproducible lots of this non-synthetic medium, however, soon became evident from the fact that chlamydospores were frequently not produced by strains otherwise fulfilling the criteria for *C. albicans*. Since determination of these other criteria requires tests of the fermentation pattern and sometimes also of animal pathogenicity, identification of *C. albicans* in the absence of chlamydospores becomes unnecessarily prolonged. Several investigators, therefore, have tried to standardize or simplify the preparation of corn meal infusion agar, or to devise other media to induce chlamydospore production in *C. albicans*.[1,2,5,9,13,16]

Of the various new media proposed, two seemed to have certain advantages over corn meal infusion agar: one, the purified polysaccharide medium of Nickerson and Mankowski,[17] because of its "known composition" and the

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fact that it is available commercially*; the second, unpurified soluble-starch agar[4] because it is simpler to prepare and is more transparent than corn meal infusion agar, permitting easier examination of the submerged mycelium.

A comparison of these 3 media for chlamydospore-inducing ability was, therefore, conducted with freshly isolated yeasts and is being reported in this paper. The procedure for identifying 179 isolates included not only determinations of morphology on the above 3 media, but also a study of their fermentation pattern by methods to be described below.

MATERIALS AND METHODS

The 3 media compared in this experiment were prepared as follows:

1. *Chlamydospore Agar (Difco).* *Rehydration of the powdered mixture with distilled water.*

2. Soluble-starch agar. Addition of 1 per cent soluble starch* (Fehling-negative) to 2 per cent agar in distilled water.

3. Corn meal infusion agar. Prepared after Benham[3] from unenriched yellow corn meal obtained at a local bakery, and modified to increase the agar concentration to 2 per cent.

The 3 media were poured while still molten in 15 to 20 ml. amounts into standard, 10 cm. Petri dishes and allowed to solidify.

To provide conditions of slightly reduced oxygen tension, the plates were inoculated by cutting running streaks into the agar with the hammered bent tip of a stiff inoculating needle, taking care not to cut all the way to the bottom of the dish. This permitted spontaneous resealing of the lips of the cut, thus preventing direct access of oxygen to the submerged inoculum. When the lips of the cut did not reseal spontaneously, they were pressed together with the needle.

Inoculum was taken from 48-hour cultures on Sabouraud's glucose agar incubated at room temperature (25° to 28° C.).

Four parallel streaks, 2 for each of 2 isolates, were accommodated per plate, those of each isolate at a distance of approximately 1 cm. from each other, and in turn 3 cm. from those of the other isolate. Each isolate was inoculated into each of the 3 media being compared. The inoculated plates were incubated at uncontrolled room temperature (25° to 28° C.) in an inverted position, and examined, still inverted, directly under the low power of the microscope after 1, 2, 4, and 7 days postinoculation. When chlamydospores were not seen, mounts were prepared to permit a systematic search of small areas with the higher magnifications required for morphologic detail. Blocks of media, approximately 7 mm.[3] containing the running inoculated cut, were placed upon slides with a drop of lactophenol mounting fluid, a cover slip applied, and the preparation heated gently. With practice the agar could be melted without boiling, the fungous elements so preserved in their original positions.

Concurrently, the carbohydrate fermentation pattern was determined using a basal broth prepared according to Martin and his associates.[11] To this basal broth, before use, 0.5 ml. of a Seitz filtered 20 per cent aqueous solution of the carbohydrate to be tested was added. In this work, C.P. glucose, maltose, sucrose, and lactose were used. Inoculum was taken from 48-hour-old cultures on corn meal infusion agar plates. Inoculated carbohydrate broths, in Durham fermentation tubes, were incubated without sealing at room temperature and examined at the end of 2, 4, 7, and 14 days postinoculation for production of acid and gas.

RESULTS

This procedure enabled us to identify 113 or 63 per cent of 179 freshly isolated yeasts as *Candida albicans.* *The remaining yeasts were identified as*

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*Chlamydospore Agar, Difco Laboratories.

(1) *C. parapsilosis*, 44 isolates or 24.5 per cent, (2) *C. tropicalis*, 5 isolates or 2.8 per cent, (3) *C. guilliermondii*, 3 isolates or 1.6 per cent, and (4) non-filamentous, asporogenous yeasts, 14 isolates or 8 per cent.

Of the 113 isolates of *C. albicans*, 109 were identified by their production of chlamydospores in at least 1 of the 3 media. The percentage of positive identification after 24 hours was 29.3 per cent on Chlamydospore Agar, 49.3 per cent on unpurified soluble starch agar, and 55.0 per cent on corn meal infusion agar, after 48 hours increasing to 68.7, 68.0, and 85.3 per cent respectively (Fig. 1). The incidence of chlamydospores increased at 4 days to 87.1 per cent on Chlamydospore Agar, 70.6 per cent on unpurified soluble starch agar, and 94.4 per cent on corn meal infusion agar, final observation at the seventh day yielded percentages of 90.9, 72.6, and 98.2 per cent respectively.

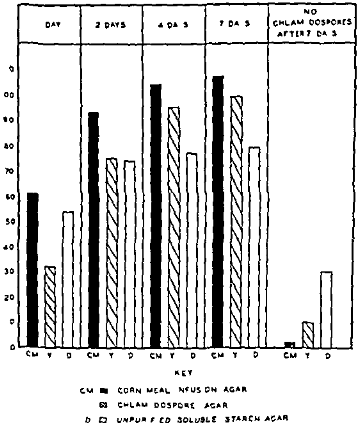

Fig. 1.—The production of chlamydospores by 109 isolates of *Candida albicans* on 3 media.

In 8 isolates diagnosis rested on the appearance of chlamydospores on one medium only: one producing them only on Chlamydospore Agar, 7 only on corn meal infusion agar. Three isolates failing to produce chlamydospores on Chlamydospore Agar did so on both corn meal infusion agar and unpurified soluble starch agar, 1 isolate failing on corn meal infusion agar was positive on both Chlamydospore Agar and unpurified soluble starch agar. Twenty-two isolates failing in the same manner on unpurified soluble starch agar produced chlamydospores on both Chlamydospore Agar and corn meal infusion agar.

Four isolates, not included in these tabulations, had failed to produce chlamydospores on any medium during the 7 day observation period. When retransferred to fresh corn meal infusion agar, 2 isolates produced chlamydospores within 24 hours. The remaining achlamydosporous isolates were identified as *C. albicans* on the basis of their microscopic morphology, carbohydrate fermentations, rabbit pathogenicity according to Benham,[3] and carbon assimilations modified after Wickerham.[17]

Fermentation reactions are defined here as the production of acid or acid and gas in the previously mentioned carbohydrate broths. Visibly detectable acid and gas were produced by 95.6 per cent of the 113 isolates on both glucose and maltose within 7 days, and by 99.1 per cent within 14 days. Acid was produced by 61.0 per cent from sucrose within 14 days; no strain, however, produced visibly detectable gas on sucrose within the same period. Further, on lactose, there was no visible production of acid and gas by any isolate.

DISCUSSION

For induction of chlamydospores in *C. albicans,* corn meal infusion agar, prepared and inoculated as described above, was superior to Chlamydospore Agar and unpurified soluble-starch agar. Not only did corn meal infusion agar induce chlamydospores in a higher percentage of isolates, but it did so in a shorter period of time.

The same medium also proved useful for the rapid morphologic identification of *C. parapsilosis, C. tropicalis,* and *C. guilliermondii.*

Even though Chlamydospore Agar is of "known chemical composition" and is available commercially, and soluble-starch agar is a simpler medium to prepare and more transparent than corn meal infusion agar, it is clear that corn meal agar continues to be the medium of choice for diagnosis of *Candida albicans.*

Since the preparation of corn meal infusion agar requires the use of a complex natural product and a prolonged period of extraction, investigators continue to try to simplify the preparation of this medium. A rapid method of extraction by boiling corn meal for 5 minutes has been used by Bakerspigel.[2] A similar rapid method of extraction has been tried by Wheeler and Little.[16] Comparison of the chlamydospore-inducing ability of these rapidly extracted corn meals with the slower method recommended by Benham,[3] and also in this paper, should be conducted before the quick-extract media can be safely used.

A more rational approach would be to continue attempts to identify what in the corn meal infusion is responsible for the induction of chlamydospores; this is being attempted in our laboratory.

Other species of the genus Candida are also known to produce chlamydospores. These are *C. tropicalis* and *C. stellatoidea.* The chlamydospores of *C. tropicalis* are, however, rarely produced, scant and atypical in morphology, and their appearance in isolates with the microscopic morphology and other diagnostic characteristics of *C. tropicalis*[3] is a reasonable safeguard against the possible misdiagnosis of this species. The chlamydospores of the so-called *C. stellatoidea,* on the other hand, are indistinguishable from those of *C. albicans.* The validity of *C. stellatoidea* as a species, however, has been seriously questioned,[10, 18] and this organism is now considered merely an avirulent variant of *C. albicans.*

SUMMARY

The morphology and conditions known to favor the induction of the chlamydospores of *Candida albicans* are described.

Three media are compared for their chlamydospore inducing ability with 109 freshly isolated strains of *C. albicans.* Corn meal infusion agar was found superior to Chlamydospore Agar (Difco) and an unpurified soluble starch agar (experimental), both as to number of isolates induced to produce chlamydospores and as to the rapidity of the induction.

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A STABLE IRON REAGENT FOR DETERMINATION OF CHOLESTEROL

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INTRODUCTION

THE iron reagent introduced by Zlatkis and co-workers[1] and MacIntyre and Ralston[2] is a sensitive and reliable reagent for the estimation of cholesterol in biologic fluids. Although Zlatkis et al.[1] states that the reagent is stable for weeks, Zak and his associates[3] indicate that the reagent has limited stability. We have found that the stock ferric chloride solution, in glacial acetic acid, develops a precipitate of ferric oxide unless the solution is kept frozen. We have also found that the color reagent, prepared by mixing stock iron solution with sulfuric acid, develops a precipitate within a few days. It occurred to us that the iron reagents could be stabilized by dissolving the ferric chloride in phosphoric acid instead of acetic acid. The iron stock solution, in phosphoric acid, was found to remain stable indefinitely at room temperature. A color reagent containing phosphoric acid was developed which remained clear for 6 to 8 weeks and our findings form the basis for this report.

MATERIALS AND METHODS

The absorption spectrum of the purple cholesterol-iron complex obtained with a Beckman D U spectrophotometer, shows a sharp absorbing maximum at 560 mμ.[1] The purple color of the cholesterol-iron complex follows Beer's law to an absorbance of 1.0 with the Sheard Sanford photometer. Other photometers are equally suitable.

Reagents.—

Iron stock solution: Dissolve 2.5 Gm. $FeCl_3 \cdot 6H_2O$ in 100 ml. of phosphoric acid (87 per cent).

Color reagent: Dilute 8.0 ml. of iron stock solution to 100 ml. with concentrated sulfuric acid (98 per cent).

Effect of Acid Concentration on Color Production.—The formation of color depends on the heat of reaction produced when sulfuric and acetic acids are mixed. The proportion of acids necessary for maximum color formation was determined by varying the ratio of sulfuric and acetic acids. The final volume, iron content, and cholesterol concentrations were maintained at optimum conditions as described under procedure. The optimum ratio of

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sulfuric to acetic acid ranged from 0.70 to 0.80 (Fig. 1). An acid ratio of 0.75 was selected, therefore, for the reaction. This ratio is at variance with the ratio of 0.65 used by other investigators.[1-3]

Effect of Iron Concentration on Color Production.—The concentration of ferric iron necessary for maximum color production was determined with color reagents containing either acetic or phosphoric acids. The concentrations of acetic or phosphoric acids were selected so that the optimal acid ratios for color formation were obtained. The various color reagents were added to cholesterol dissolved in 5 ml. of acetic acid as given in the procedure.

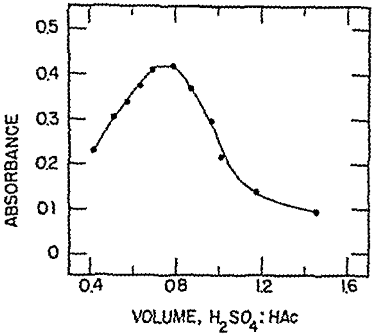

Fig. 1.—The ratio of sulfuric acid to acetic acid versus absorbance of color formed. All reactions were performed in a final volume of 9 ml.

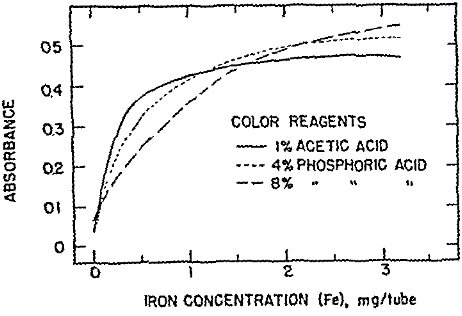

Fig. 2.—The concentration of iron versus absorbance of color formed, in the presence of varying amounts of acetic acid and phosphoric acid.

The formation of color increases rapidly as the concentration of iron increases (Fig. 2). With the color reagent containing acetic acid, very little additional color is formed at high concentrations of iron. The substitution of phosphoric acid for acetic acid in the color reagent decreases the availability in Table II. A comparison study of the new iron reagent and the Lieberman-Burchard reaction on serum samples prepared by the procedure of Sperry and Webb,[4] also show satisfactory agreement (Table III).

SUMMARY

A new, stable iron reagent that yields results comparable to other procedures and eliminates the need for preparing color reagents at more than infrequent intervals is described.

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NEW, RAPID, AND PRACTICAL MICROMETHODS FOR PROTHROMBIN DETERMINATION

I The Watch Glass Method

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The introduction of the one stage prothrombin time test by Quick initiated the remarkable advances in the study of blood coagulation and its abnormalities over the last 15 years The test is widely used today as the best test for the control of anticoagulant therapy and is as well an important tool in coagulation research studies

While possessing many merits, the test does have certain disadvantages The proper performance of the test usually requires that the patient be in the hospital, have accessible veins for puncture, and that the test itself be carried out under relatively standard conditions in the hospital laboratory

It would be advantageous for the physician to have this very useful test available at the bedside, in the operating room, or in his private office Further, the avoidance of repeated venipuncture and the practical possibility of an immediate result would make the test even of greater value both clinically and experimentally The one stage test as performed today does not include these desirable features Consequently, we have modified the Quick prothrombin time test to produce, in effect, two micro tests for obtaining accurate and immediate results utilizing one or two drops of blood obtained by simple finger stick or pick up from oozing or injured surfaces

It is the purpose of this paper to describe one of the two tests and to report the results obtained with it on over 100 patients receiving anticoagulant therapy, as well as to compare the results with simultaneously obtained Quick one stage values Another micromethod, the so called "Hematocrit method" is reported elsewhere in this Journal

MATERIALS

White blood cell pipettes of the usual type were employed

Watch glasses, glass diameter = 6 5 cm were used These were acid washed and not siliconized

Thromboplastin, prepared as previously described[1]

Stop watch, an ordinary hand stop watch was employed

Lancets were used for finger stick

METHODS

Performance of the Test—(Fig 1) Thromboplastin is drawn to the "1" mark of a white blood cell counting pipette and placed in a watch glass An equal amount of blood

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is drawn with a second pipette from a freely flowing finger stick and blown into the thromboplastin, and immediately a stop watch is started. Mixing of the two may be enhanced by gently blowing through the pipette for a few seconds. *After a pause of ten seconds,* the watch glass is tilted every second and observed over a white paper or a flashlight until fibrin strands or gel formation sets in. This is a clear-cut end point. The room temperature is noted.

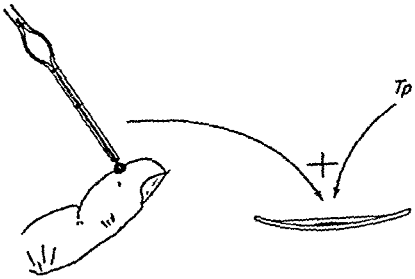

Fig. 1.

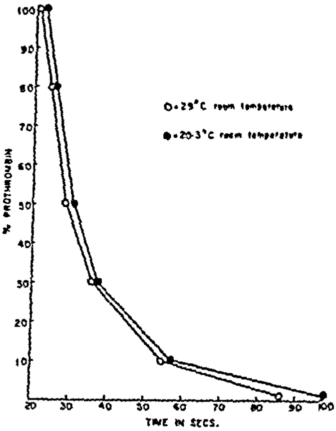

Fig. 2.

Fig. 1.—The essential steps in the performance of the Watch Glass test are illustrated. After simple finger stick with a Hemolet, the first few drops of blood are wiped away and blood is drawn to the 1 mark of the white blood cell pipette. This is quickly mixed with an equal amount of thromboplastin already contained in the watch glass. At the time of mixture, a stop watch is started. The end point is clear-cut. The room temperature is noted.

Fig. 2.—Dilution curves, Watch Glass method. Shown is the dilution curve used to convert clotting times to "per cent prothrombin." This is a function of the room temperature, the thromboplastin preparation, and the blood sample. Values may be interpolated for room temperatures in between those shown in the figure.

Conversion of Clotting Time to "Per Cent Prothrombin."—A standard dilution curve similar to that of Quick was plotted by determining the clotting times of whole bloods of varying "per cent prothrombin" which were artificially prepared. This latter was simply accomplished in two steps: first, obtaining various "per cent prothrombin plasmas" by mixing aluminum-absorbed plasma (so-called prothrombin free plasma) and normal plasma in various proportions. Then, by mixing such plasmas with their well-washed cellular components in the proportion indicated by their hematocrit values, varying per cent prothrombin whole bloods were prepared. The standard dilution curves obtained at two extremes of room temperature are shown in Fig. 2.

RESULTS

In Fig. 3 is seen a comparison of results obtained on blood from over 100 patients, both normal and receiving anticoagulant therapy, with the Quick test and the Watch Glass test. The values are distributed around the theoretical regression line, $y = ax + b$ where $a = 1.00$ and $b = 0$. The least squares regression formula for the observed values is $Y = 1.019\ X + 0.16$. P value in a test of significance for linear regression of observed versus theoretical is greater than 0.9. The standard deviation of the Quick one-stage method in our laboratory was found to be 0.3 second at the 100 per cent prothrombin level This is equivalent to 6 per cent "prothrombin" The standard deviation of the Watch Glass method in our laboratory was 0 9 second This is equivalent to 4 5 per cent "prothrombin"

DISCUSSION

The data obtained comparing the Watch Glass method with the Quick method for "prothrombin" determination on 108 patients are in good agreement with the theoretical values The variations are quite comparable and the relative errors are also comparable Thus the data are judged acceptable on a statistical basis From a practical viewpoint, the test has proved quite helpful when anticoagulant therapy was needed on a patient whose veins were not palpable Moreover, its usefulness in pediatric cases or where repeated sampling is needed seems evident

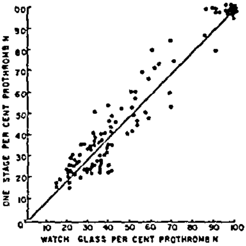

Fig 3 —Correlation of prothrombin determinations The scattergram represents the results obtained with 108 patients both normal and receiving anticoagulant therapy Prothrombin values by the Quick method and the Watch Glass method are plotted The line of perfect correlation is indicated The values obtained by both methods are statistically comparable

Finally, assuming suitable controls are employed and a stable thromboplastic preparation is available, the method is adaptable to a bedside procedure, an office procedure, a pediatric procedure, or a procedure on patients without accessible veins

SUMMARY

A new micromethod for prothrombin determinations is described Results on over 100 patients with the Quick test and the Watch Glass method are presented The usefulness of the test is discussed

We wish to thank Miss Joyce Black for technical assistance

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NEW, RAPID, AND PRACTICAL MICROMETHODS FOR PROTHROMBIN DETERMINATION

II. THE HEMATOCRIT METHOD

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THE need for micromethods of prothrombin estimation has been variously met in the past. Quick,[1] in 1939, reported a very simple bedside technique employing a drop of capillary blood and a drop of thromboplastin on a slide; he mixed and then watched for a suitable end point. (In this paper he criticized Smith and his associates for their proposed formula.) In 1940, Kato[2] employed capillary blood, oxalated it upon drawing, and thus required in the test the addition of calcium. His method correlated well with the Quick method; however, the procedure is long, very exacting, and requires considerable equipment for a "rapid" bedside technique. In 1941, Innes and Davidson[3] collected capillary blood in white blood cell counting pipettes, oxalated the blood in a watch glass, and then mixed it with snake venom. In 1942, Ulin and Barrows[4] described a new method employing a wire loop coagulomotor and capillary blood. Lufkin and Strolber,[5] using calibrated pipettes, delivered blood and thromboplastin in proportion of 1:4 into a watch glass. These last three methods all expressed results using the formula of Smith and associates. However, none of these methods has been widely accepted.

In the course of work, our laboratory was requested to contrive some manner of following anticoagulant therapy on a patient with no accessible surface veins. Further, the Watch Glass method[6] had not yet been adequately investigated and standardized. Such a method was devised, utilizing no principles other than those of the Quick one-stage procedure, save that only a few drops of whole blood from a finger stick served as the sample. It is the purpose of this paper to describe the method, the results obtained therewith, and its use as a special, though not difficult, laboratory test.

MATERIALS

Wintrobe hematocrit tube
Pasteur pipettes with long stem
Alumina-adsorbed plasma[8]
Thromboplastin (prepared as previously described[7])
Stop watch
Lancets
Test tubes, water bath[6]
Silicone, D.C. 200* was used as a 2 per cent suspension in CCl_4
Glassware so treated was dried in hot-air oven at 190° C. for 1 to 2 hours.

From the Sidney Hillman Medical Center, Philadelphia, and the Hahnemann Medical College and Hospital of Philadelphia.
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*Kindly supplied by the Hollingshead Company, Camden, New Jersey.

METHODS

Performance of the Test.—(Fig. 1.) The essential principle in this method is that the test sample of whole blood from a finger stick is received into a siliconized Wintrobe hematocrit tube which contains a known amount of "deprothrombinized" (alumina-adsorbed) plasma which is slightly overoxalated. After centrifugation, the additional test plasma volume may be simply calculated by reading the increase in total volume and subtracting the red cell volume at the bottom of the tube. Therefore, if the test sample contained 100 per cent "prothrombin," the plasma mixtures should give a "per cent prothrombin" value in a one-stage test equal to the per cent test plasma. Values of per cent prothrombin in the test plasma may be calculated by the formula:

$$\frac{\text{Per Cent Prothrombin Values of Mixture}^{*}}{\text{Per Cent Test Plasma in Mixture}^{\dagger}} \times 100 = \text{"Per Cent Prothrombin in Test Plasma"}$$

It will be immediately recognized that this is the procedure for making prothrombin clotting time calibration curves and thus employs no new principles in the one-stage test (Fig. 2.) A typical detailed procedure follows: 4.5 ml. normal human blood was collected in a test tube containing 0.5 ml. of 0.1 M oxalate. The plasma obtained by centrifugation was "deprothrombinized" by alumina gel adsorption. Its clotting time after adsorption was more than four minutes. Exactly 0.50 ml. of the alumina adsorbed plasma was placed in a clean siliconized Wintrobe hematocrit tube with a long-stemmed Pasteur pipette after 0.02 ml. of 0.1 M oxalate was added. The volume was then precisely read and noted to be 0.52. A few drops of blood from a clean finger stick were taken up in a siliconized long-stemmed Pasteur pipette, placed in the Wintrobe tube, and carefully and intimately mixed with the alumina plasma sample. After centrifugation, the total volume was read and noted to be 0.75 ml. The red cell volume was read and noted to be 0.10 ml. The additional test plasma was then calculated to be 0.13 ml. and present in the plasma mixture

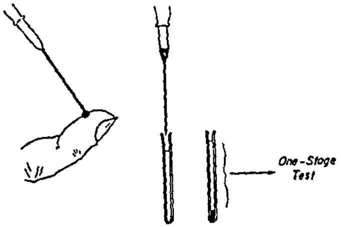

Fig 1

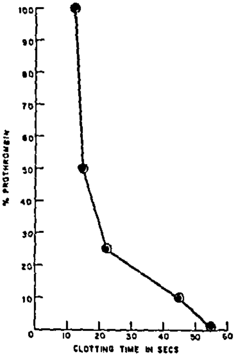

Fig 2

Fig 1—This figure illustrates the essential steps in the performance of the Hematocrit method for "prothrombin" estimation After simple finger stick with a Hemolet, the first few drops of blood are wiped away, and blood is drawn into a siliconized Pasteur pipette A convenient amount is then added to a Wintrobe hematocrit tube containing a known amount of slightly overoxalated, "prothrombin-free" plasma and mixed well After centrifugation the total volume and red cell volume are noted The supernate plasma is then subjected to a regular Quick (prothrombin time) test. Calculation of per cent prothrombin in the patient's blood sample is described in the text

Fig 2—One-stage curve Shown is the dilution curve used to convert the clotting times of the plasma mixture to 'per cent prothrombin" Calculation of the per cent prothrombin in the patient's blood sample is described in the text

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*Obtained by converting clotting times of mixture to per cent prothrombin
†Obtained by noting volumes in the hematocrit tube

as 23 per cent (0.13 + 0.2/0.65). The clotting times obtained were 36.8 and 37.5 seconds. When the plasma mixture was analyzed by the conventional one-stage method, this was read to be equal to 15.5 per cent. Thus, the per cent prothrombin in the test plasma was calculated to be: $\frac{15.5}{23} \times 100 = 67$ per cent.

RESULTS

In Fig. 3 is seen a comparison of results obtained on blood from over 100 patients, both normal and receiving anticoagulant therapy with the Quick test and the Hematocrit test.

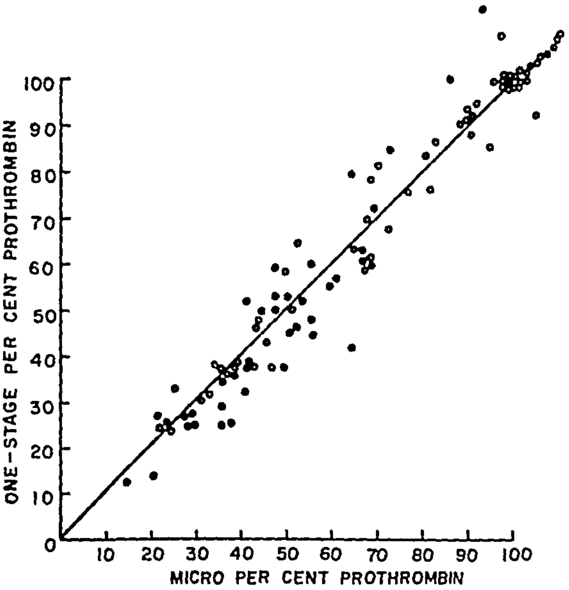

Fig. 3.—Correlation of prothrombin determinations. Shown are the results obtained with 108 patients, both normal and receiving Prothrombin values by the Quick method and the Hematocrit meth line of perfect correlation is indicated. The values obtained by both methods are statistically comparable.

The values are distributed around the theoretical regression line, $y = ax + b$ where $a = 1.00$ and $b = 0.00$. The least squares regression formula for the observed values is $Y = 0.9646\ X + 0.37$. P value in a test of significance for linear regression of observed versus theoretical is greater than 0.9. Standard deviation = 8 per cent. The standard deviation of the Quick one-stage method in our laboratory was found to be 6 per cent. The standard deviation of the Hematocrit method in our laboratory was thus comparable.

DISCUSSION

The data obtained comparing the Hematocrit method with the Quick method for "prothrombin" determination on 108 patients are in good agreement with the theoretical values. The variations are quite comparable and the relative errors are also comparable. Thus, statistically, the data are judged acceptable.

A word should be said in interpretation of these results compared to the interpretation of the regular Quick one stage results. It is widely accepted that the one stage test is an integrated measure of the nonthromboplastic components of the coagulation mechanism. However, a normal clotting time in this micro one stage test indicates a gross adequacy of only those components which were removed by the alumina adsorption: prothrombin and pro SPCA (serum prothrombin conversion accelerator). Thus, this particular modification of the one stage test, employing as it does an alumina plasma menstruum, may be said to differ from the regular one stage test in that it provides a measure of prothrombin and pro SPCA only.

SUMMARY

This test is practical and has proved helpful in certain clinical cases. Like the Watch Glass test, its value in patients "without veins," in pediatrics or repeated samplings seems obvious. Further, its use in experimental procedures, for example, in small animal experimentation, would also seem advantageous.

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A METHOD FOR ESTIMATING THE BACTERIAL POPULATION OF THE OROPHARYNX

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IT IS difficult to quantify the bacterial flora of the oropharynx. This difficulty was encountered by us in a study which involved the daily variation of the bacterial population of the throat The methods commonly used provide only a rough quantitation of the oropharyngeal bacteria.[1-6] Since the accuracy of the procedures in use have not been established, certain technical refinements seem essential in order to insure a more reliable analysis of the bacterial population of the throat

The actual number and type of bacteria in the pharynx and their precise in vivo relationships to each other probably cannot be determined. For example, one cannot estimate the actual number of streptococci present in the oropharynx since the total quantity of pharyngeal secretions from which the counted aliquot is obtained cannot be determined In addition, the growth of the various species of bacteria in vitro depends upon the medium used, the period of incubation, the O_2 and CO_2 tensions, and other environmental conditions

The present investigation was limited to an evaluation of the aerobic streptococci and *Staphylococcus albus* in relation to other bacteria present in the oropharynx and as this is reflected on the specific cultural environment we used. A statistical analysis was then applied to determine the limits of accuracy of the technical procedures.

MATERIALS AND METHODS

A platinum inoculating loop was sterilized, cooled, and then rubbed across the surface of the oropharynx with care to avoid the uvula and tongue The loop was then agitated in 1 ml of sterile physiologic saline The saline was immediately poured onto the surface of a 5 per cent sheep blood agar plate Trypticase soy agar was the basal medium The plate was rotated to distribute the solution evenly over the surface and then incubated for 48 hours at 37° C

Colony counts were made in the following manner· a section of the plate was arbitrarily selected and 200 or more of all types of colonies were counted and grossly identified Counting was done with the aid of a hand lens. Verification of the species was accomplished by Gram stain and subculture to appropriate media when necessary. The percentage of each species was then calculated

Two methods were used to test the reliability of the procedure First, the wire loop was immersed in the same 2 ml of saline after each of two swabbings of the patient's oropharynx The sample was divided into two parts and each ml. sample was then plated.

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The two plates were treated as described above. Twenty healthy persons (hospital personnel) were tested in groups of 3 and 4 at a time. The plates were coded by a person other than the technician who did the counting in order to conceal the identity of the pairs.

In the second experiment, two consecutive cultures were taken from each person. The initial swabbing was introduced in 1 ml. of saline. The loop was sterilized over a flame, allowed to cool and the second swabbing was carried out in a similar manner and introduced into another ml. of saline. Each sample was cultured as described above. Approximately 2 to 3 minutes elapsed between taking the two independent, consecutive samples. These plates were also coded. Nineteen of the subjects used in the previous experiment were tested in this manner.

RESULTS

Alpha and gamma streptococci and *Staphylococcus albus* percentages were obtained. The results of the experiments are shown in Table I.

Single Sample Divided.—When the single samples were divided into two parts, the ninety-five per cent confidence statements, as determined by an analysis of variance technique, were found to be $x - 3.2 \leq \xi \leq x + 3.2$ for gamma streptococci and $x - 3.8 \leq \xi \leq x + 3.8$ for alpha streptococci, where x is observed per cent count and ξ is the "true" per cent, and where the distribution for per cent counts is assumed to be approximately of Gaussian form. When the alpha and gamma are added to obtain a total streptococcal count and then analyzed in a similar manner the ninety-five per cent confidence statement is $x - 3.9 \leq \xi \leq x + 3.9$. These values are in terms of per cent of the species of bacteria of the total of over 200 colonies of all species counted on the plate. The *Staphylococcus albus* counts are also shown in Table I, however, the large number of samples with no growth of this organism precludes the assumption of Gaussian distribution.

Two Consecutive Samples.—The ninety-five per cent confidence statements in the experiment where two independent samplings of the oropharynx were obtained at a 2- to 3-minute interval of time were $x - 2.9 \leq \xi \leq x + 2.9$ for gamma streptococci and $x - 5.2 \leq \xi \leq x + 5.2$ for alpha streptococci. For total streptococci the confidence statement was $x - 3.5 \leq \xi \leq x + 3.5$. For the reason mentioned in the previous paragraph, no confidence limits were determined for *Staphylococcus albus* counts. They are, however, listed in Table I.

Beta hemolytic streptococci were not isolated in any of the cultures, possibly because the cultures were obtained during the summer months. Other bacteria isolated included Neisseria (chiefly *N. catarrhalis*), diphtheroids, *Staphylococcus aureus, Hemophilus parainfluenzae,* and *Diplococcus pneumoniae.*

DISCUSSION

The first experiment, which involved dividing a single sample into two parts and culturing and counting each separately, provides limits of the reliability of the procedure after the sample has been taken from the oropharynx. These limits are roughly 3 plus per cent on either side of the true count for alpha and gamma and total streptococci. The second experiment which involved consecutive samples, taken at about 2 minute intervals, gives a further index of the added error in the swabbing procedure. This, however, would assume that no

TABLE I

SUBJECTS	ONE SAMPLE DIVIDED INTO TWO PARTS (PER CENT COUNTS)						DAYS BETWEEN EXPERIMENTS	TWO CONSECUTIVE SAMPLES (PER CENT COUNTS)					
	GAMMA STREPTOCOCCI		ALPHA STREPTOCOCCI		STAPHYLOCOCCUS ALBUS			GAMMA STREPTOCOCCI		ALPHA STREPTOCOCCI		STAPHYLOCOCCUS ALBUS	
1	6.9	7.0	62.5	61.8	4.9	5.1	23	1.4	0.9	83.8	78.6	1.4	1.8
2	12.3	11.0	52.6	52.6	0.9	0.0	23	3.9	4.8	65.4	66.7	0.5	0.0
3	10.5	13.6	4.1	6.6	0.0	0.0	21	21.5	21.5	38.5	35.4	1.4	0.6
4	92.4	92.2	5.6	5.4	0.0	0.0	21	57.1	56.5	25.7	25.6	0.3	0.3
5	52.5	51.2	39.6	35.5	1.3	1.2	79	38.8	36.0	53.6	50.8	0.9	1.1
6	80.5	79.5	18.0	18.6	0.0	0.6	75	15.5	16.0	24.1	25.3	0.5	0.4
7	85.5	85.1	13.2	13.6	0.3	0.3	16	30.6	29.9	56.9	59.2	1.2	1.9
8	27.0	26.8	45.6	46.3	2.1	1.6	16	47.1	47.6	39.0	38.1	0.7	2.4
9	19.0	23.0	66.7	64.7	1.6	1.4	15	14.9	19.3	59.8	69.1	0.4	0.0
10	3.2	2.3	66.0	73.4	0.0	0.0	15	5.2	7.9	64.8	61.1	1.1	0.9
11	21.0	22.5	64.8	60.7	2.5	0.5	15	16.5	15.9	53.6	54.2	0.3	0.3
12	34.2	27.9	53.9	55.7	0.7	0.4	70	15.2	17.9	70.1	66.1	0.3	0.0
13	4.1	5.6	52.2	55.2	4.1	5.0	70	30.0	29.8	45.0	46.5	0.5	0.0
14	35.2	37.4	21.4	20.9	0.0	0.5	0	39.3	35.4	17.0	22.0	0.0	0.0
15	14.0	13.3	50.8	48.1	0.0	0.4	0	14.4	15.6	51.7	50.6	0.3	0.3
16	65.3	66.9	19.5	19.1	0.0	0.0	0	61.6	61.3	20.5	21.5	0.0	0.8
17	28.7	30.3	33.1	33.8	0.8	0.3	0	25.7	26.5	39.3	38.1	1.5	0.9
18	10.8	10.8	28.1	26.6	0.0	0.0	0	13.8	10.5	21.3	27.0	0.0	0.0
19	46.4	44.1	42.2	42.6	0.0	0.0	15	22.2	23.1	53.0	52.4	1.3	1.1
20	21.0	22.5	49.5	46.1									
95 per cent confidence statement*	$x - 3.2 \leq \xi \leq x + 3.2$		$x - 3.8 \leq \xi \leq x + 3.8$					$x - 2.9 \leq \xi \leq x + 2.9$		$x - 5.2 \leq \xi \leq x + 5.2$			

*Where x is the observed per cent count and ξ is the "true" per cent count.

change occurred in the flora during the intervening 2 minutes There was no difference in the limits of error for the divided single sample and the two consecutive samples for gamma streptococci However, there was some increase in error in consecutive sampling for alpha streptococci Total streptococci remained the same in both cases and essentially averaged out the gamma and alpha counts In any event, it is apparent that estimates of these bacteria in the oropharynx can be made with some precision There was not a significant regression of the magnitude of discrepancy between the two samples on the size of the count This suggests that the estimated variance applies approximately to large or small percentage counts alike All counts were done by the same technician however, and do not preclude any consistent errors of identification she could have made

We were interested in the variation of the counts when two swabs were taken independently but within a short span of time as was done in the second experiment The results indicate that the flora do not vary greatly from moment to moment and that the two separate swabbings pick up essentially the same distribution of these bacteria Some further observations can be made of changes that occur in the percentage counts over longer periods of time This can be done by comparing the values obtained on each subject for both experiments The same subjects were used for both experiments and the time that elapsed between the experiments varied The interval was approximately 3 weeks for the first 4 subjects, 2½ months for the fifth and sixth, 2 weeks for Subjects 7 to 11, and 2 months for Subjects 12 and 13 Both experiments were done on the same day and within the same hour for Subjects 14 to 18 but there was a lapse of 2 weeks for Subject 19 The changes were minimal when both tests were done within the same hour (Subjects 14 to 18) Examples of both increase and decrease in percentage of these bacteria were found when the time interval extended from 2 weeks to 2½ months

It is difficult to interpret the results of the *Staphylococcus albus* counts They show some degree of reproducibility but the persistently low percentage range magnifies the differences between two samples It would appear that any attempt to follow daily variations would be inaccurate unless the counts ranged into higher values

Ordinarily, cotton swabs have been used to obtain samples from the oropharynx The swabs are then streaked directly onto the surface of the medium We found that this technique causes overgrowth and crowding of the colonies In addition, the possibility of trapping the organisms in the cotton fibers exists Some investigators have introduced the swab into broth and mixed by repeated pipetting of the broth[3, 5] We found that an ample number of organisms could be picked up on a platinum inoculating loop and although we could not be certain, we felt that there was a better possibility for transport of organisms into the solution Practically all of the subjects felt that this procedure was less uncomfortable than sampling with a cotton swab Flooding the resulting diluent across the surface of the medium provided good distribution of colony growth It reduced crowding and provided good conditions for identification and counting. One milliliter of diluent and 48-hour incubation provided optimal conditions for counting the bacterial colonies that were of particular interest to us. Sheep blood agar was superior to human blood agar for differentiation of hemolytic and nonhemolytic streptococci.

SUMMARY

Some refinements of the technique of estimating the streptococcal and staphylococcal population of the oropharynx are described. Statistical analysis of the data derived by the use of these refinements indicates that the oropharyngeal flora can be estimated in more precise terms than heretofore described.

We wish to acknowledge with gratitude the assistance of Malcom Turner, Biostatistics Department, Kettering Laboratory, and Dr. Dorothy Fleming, Department of Microbiology of the University of Cincinnati College of Medicine.

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THYROID CLEARANCE, UPTAKE, AND RATE OF UPTAKE OF RADIOIODINE IN HYPERTHYROIDISM

Their Interrelationships and Diagnostic Value

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SINCE the early investigations[1, 2] almost twenty years ago which demonstrated the effectiveness of radioiodine as an indicator of thyroid function, numerous reports have appeared proposing various techniques for appraising thyroid activity. Many of these have been discarded for practical reasons. The thyroid I¹³¹ uptake has been the most universally used test, although the optimum time to measure the uptake after a tracer dose has not been agreed upon. The rate of thyroid uptake has been studied but not applied generally as a means of diagnosis.[3] Several investigators[4-6] have claimed the thyroid I¹³¹ clearance to be the most reliable and sensitive indicator of the state of thyroid function. Because of the diverse views as to the most effective means of diagnosing thyroid dysfunction by radioiodine tracer techniques, clarification is desirable.

The present study is concerned with three measures, thyroid uptake, thyroid clearance, and rate of thyroid uptake, attempting to evaluate their relative merits and interdependence as they are used clinically in the diagnosis of hyperthyroidism.

MATERIALS AND METHODS

Patients.—The euthyroid group consisted of 109 hospital patients without goiter or clinical evidence of thyroid disease. All but 2 were males. The age range was 21 to 66 years.

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The hyperthyroid group consisted of 50 patients with unequivocal clinical hyperthyroidism. Forty-four had diffuse and 6 had nodular goiters. Only 3 were females. The age range was 20 to 66 years.

In addition to the clinical findings, the basal metabolic rate and the serum chemical protein-bound iodine concentration were utilized to support the clinical impression in both groups. None of the subjects were receiving medications known to affect thyroid function, and none had cardiac or renal diseases, or disorders which indirectly might influence the test results.

Measurements.—All the tests to be described were performed simultaneously on each patient in the fasting state following an oral dose of 150 microcuries of I^{131}. *Thyroid uptake* measurements were made ½, 1, 2, 3, and 24 hours after the tracer dose, using a technique described previously.[7] *Thyroid clearances* of I^{131} were calculated for the time periods ½-1, 1-2, and 2-3 hours after administration of the tracer dose. Thyroid clearance was calculated as the ratio of the amount of radioactivity taken up by the gland in a given time interval to the average plasma concentration of radioactivity during that interval. The actual formulation used was

$$TC = \frac{U_2 - U_1}{(P_1 + P_2)/2} \times \frac{1}{t_2 - t_1}$$

where U_1 and U_2 are the percentage thyroid uptake at times t_1 and t_2; $(P_1 + P_2)/2$ is the average plasma I^{131} concentration in percentage of the dose per milliliter during time interval $t_2 - t_1$. This arithmetic average of plasma concentrations is used as an approximation to the true average, $\frac{1}{t_2 - t_1}\int_{t_1}^{t_2} P\,dt$, derived from the exponential curve of plasma concentration. The true average is slightly overestimated by the arithmetic mean, giving clearances that are correspondingly underestimated. Since with time this discrepancy decreases progressively, the early clearances tend to be underestimated slightly more than those that follow. TC is thyroid clearance expressed as milliliters of plasma cleared of I^{131} per minute. For the calculation of the ½-1 hour clearance the plasma I^{131} concentration at one hour was taken as an approximation to the average concentration during the interval ½-1 hour, $t_2 - t_1$ being taken as 30 instead of 60. The methods used for counting plasma I^{131} have been described previously.[7]

Rate of thyroid uptake was calculated from curves based upon five observed points, ½, 1, 2, 3, and 24 hours after a tracer dose. Only five points were used in plotting the curve to simplify the procedure as it might be used in clinical work. The mean rates of uptake in the present study were very similar to those reported by Luellen, Keating, and co-workers[3] in euthyroid and hyperthyroid subjects, using more points to determine the curve of thyroid uptake.

The rate at which the maximum uptake was approached was calculated from the equation, $U_t = U_m\,(1 - e^{-kt})$ where U_t is the percentage thyroid uptake at any time t, U_m is the maximum percentage uptake, taken to be the 24-hour value in this study, and k is the rate constant.[3] Though we recognize that the 24-hour uptake is often not the maximum value in hyperthyroid patients, it usually does not deviate greatly from the true maximum. In euthyroid patients the 24-hour uptake is almost invariably higher than any earlier value.

The rate, k, was determined by solving the above equation in its logarithmic form rearranged for convenient calculation, $\log\,(U_m - U_t) = \log U_m - kt$. This simply represents a plot on semilogarithmic paper of the differences between uptakes during the first 3 hours and the maximum uptake. If one arbitrarily plots in similar fashion the differences between uptakes during the first 3 hours and the dose given, one also gets a straight line whose slope reflects the rate at which the uptake by the gland during this time interval approaches the dose administered. This we have called the rate to dose. Though an arbitrary calculation, it has the merit of a single reference point (the dose given) for both hyperthyroid and euthyroid patients, and as a consequence the measure takes into account differences in final uptake achieved by the two types of patients as well as the differences in rate of uptake by the gland. As will be seen, it has particular value for discriminating between euthyroid and hyperthyroid patients, even though it has no general meaning in terms of the whole curve of uptake by the gland These two rates were calculated for each patient, being expressed as the percentage of the maximum or dose taken up per hour during the time interval studied.

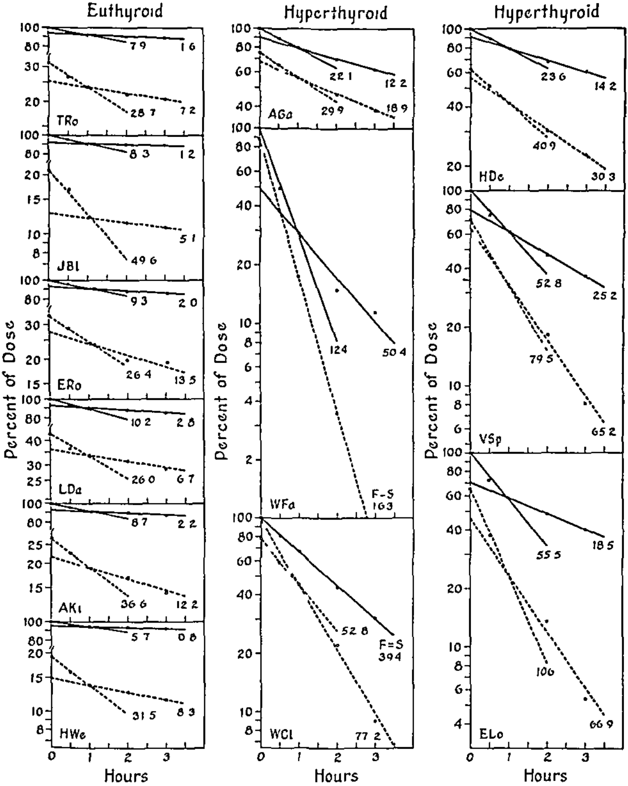

Fig 1 —Semilogarithmic plots used in calculating rates of uptake of radioiodine in 6 euthyroid and 6 hyperthyroid patients Solid lines used in calculating rates to dose broken lines rates to maximum Number recorded at the end of each line is the slope of the line which is the rate constant in per cent of the dose per hour

In plotting $(U_m - U_t)$ on semilogarithmic paper it was observed that except in some severely hyperthyroid patients two components were evident The first, a rapid component, occurred during the first hour and reflected the increasing blood concentration resulting from absorption of the oral dose during this interval The second slower component reflected the decreasing blood concentration which was apparent after the first hour. Representative examples of the semilogarithmic plots of $(U_m - U_t)$ against time are given in *Fig. 1* for 6 euthyroid and 6 hyperthyroid patients. The two components were evident whether the rate to the maximum (broken lines) or the rate to the dose (solid lines) was plotted. Among the hyperthyroid patients, whose rates of uptake were faster, the change in slope on going from the first to the second component tended to be less than among euthyroids. An occasional severely hyperthyroid patient had an uptake curve adequately represented by a single exponential and did not show two components on the semilogarithmic plot. In such a case the first (fast) and second (slow) components were equal as in the rate to maximum curve of Patient WFa, and the rate to dose curve of Patient WCl. Very rarely a hyperthyroid patient showed a slower first than *second* component as in the rate to maximum curve of Patient WCl.

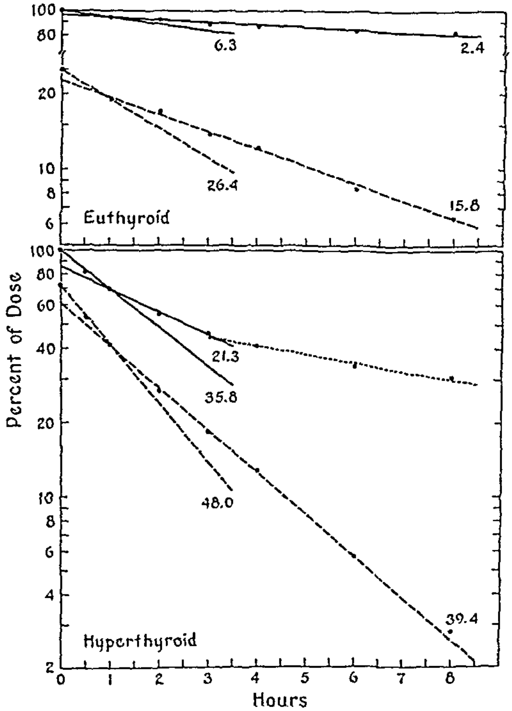

Fig. 2.—Semilogarithmic plots used in calculating rates of uptake of radioiodine for the data summarized in Fig. 4 of the paper of Luellen, Keating, and co-workers.[3] Remainder of legend as in Fig. 1.

These two components of the rate curves are apparent in the data of Luellen, Keating, and associates[3] as shown in Fig. 2 reconstructed from their average curves (assuming, for purposes of plotting, an average 24-hour uptake of 72 per cent for the hyperthyroids and 25 per cent for the euthyroids). A single exponential adequately represents the slow component of the curve among their euthyroid patients during the time interval from 1 to 8 hours whether one plots the rate to maximum or rate to dose. A similar observation is evident for the rate to maximum curve of the hyperthyroid patients but not for the rate to dose curve, which requires a third exponential to account for the segment beyond three hours, as indicated by the dotted line.

Analysis.—Frequency and cumulative frequency distributions were plotted for each variable and each group of patients. In plotting the frequency graphs, the normal range of each test was subdivided into five class intervals. Thus, in terms of class intervals the distance along the abscissa of each graph is identical from test to test for the euthyroid subjects, and the spread of hyperthyroid values may be compared. Means and standard deviations are recorded for each distribution.

Intercorrelations among the measures were calculated for the 50 hyperthyroid patients and for 50 euthyroid subjects selected at random from the larger group.

RESULTS AND INTERPRETATIONS

Thyroid Uptake.—Although there was effective separation of the euthyroid and hyperthyroid groups at all time intervals studied (Fig. 3), the best separation of the two groups occurred at 3 hours. Uptakes within the first 3 hours after an oral tracer dose were superior to the 24-hour uptakes, as evidenced by wider separation of the means for the two groups, slightly less overlapping, and less compression of the range of values of the hyperthyroid group. The mean uptakes at 3 hours were 10 ± 3 per cent and 58 ± 16 per

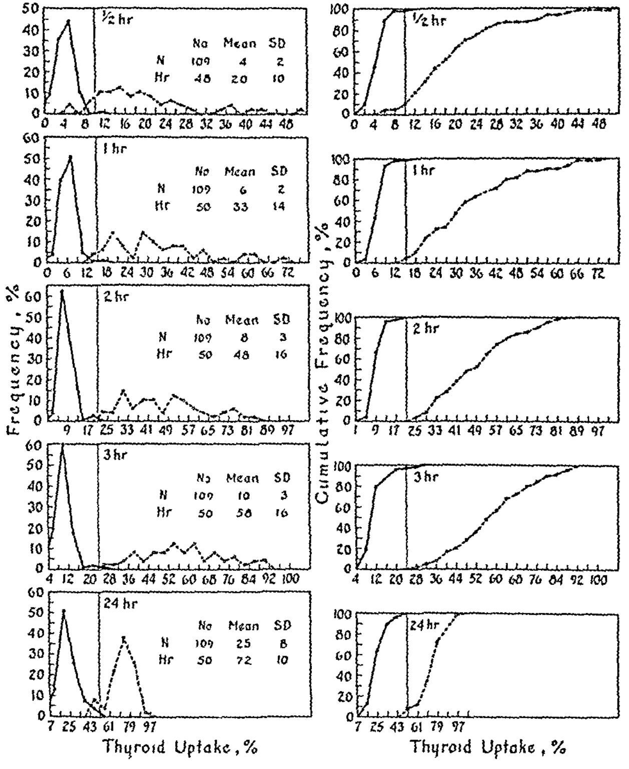

Fig 3.—Frequency distributions of thyroid uptake of radioiodine at various time intervals by group Solid line represents euthyroid or normal (N) subjects Broken line represents hyperthyroid (Hr) patients

cent for the euthyroid and hyperthyroid groups, respectively. At 24 hours the mean values were 25 ± 8 per cent for the euthyroid group and 72 ± 10 per cent for the hyperthyroid group.

At 3 hours, one-half of the euthyroid subjects had uptake values less than 10 per cent and 96 per cent had values under 20 per cent. One-half of the hyperthyroid group had values exceeding 57 per cent, and 96 per cent exceeding 32 per cent. At 24 hours, 90 per cent of the euthyroid group had uptake values under 35 per cent and only one patient had a value above 50 per cent. Ninety-six per cent of the hyperthyroid group had uptake values above 56 per cent, and only one patient had a value below 50 per cent.

Based upon our sample of 109 euthyroid subjects, we would set the upper limits of normal for thyroid uptake at 1, 2, and 3 hours after a fasting oral dose as 15, 20, and 25 per cent, respectively.

Thyroid Clearance.—Among the hyperthyroid patients, the thyroid I^{131} clearance decreased rapidly in the first 3 hours, as a rule, as indicated by the mean values in Fig. 4. This was also true for the euthyroid group, but less apparent because of the smaller clearances. The percentage decrease in clearance was similar for the two groups. Between the intervals, ½-1 hour and 1-2 hours, there was a decrease in clearance of 45 per cent for the euthyroid group and of 34 per cent for the hyperthyroid group. Between the 1-2 hour and 2-3 hour intervals, the decreases in clearance for the euthyroid and hyperthyroid groups were 10 per cent and 14 per cent, respectively.

Separation of euthyroid and hyperthyroid patients was good at all time intervals studied (Fig. 4). There was slightly more overlapping at ½-1 hour than at later intervals; however, there was little to choose between the 1-2 hour and 2-3 hour periods. The mean thyroid I^{131} clearances at the various time intervals are indicated in Fig. 4.

At 2-3 hours, 50 per cent of the euthyroid I^{131} clearances were below 8 ml./min., 90 per cent were less than 19 ml./min., and 96 per cent were less than 25 ml./min. Fifty per cent of the hyperthyroid I^{131} clearances were above 120 ml./min., 90 per cent were above 42 ml./min., and 96 per cent were above 30 ml./min.

Based upon the sample of 109 euthyroid subjects the upper limits of normal for thyroid I^{131} clearance at ½-1, 1-2, and 2-3 hours may be set at 40, 30, and 25 ml. per minute, respectively.

Rate of Thyroid Uptake.—As one might expect, the rate at which the dose was taken up was superior to the rate at which the maximum uptake was reached, for purposes of discriminating between euthyroid and hyperthyroid patients. Likewise in each instance, the rate for the slow component was more discriminative than the rate for the rapid component. Thus the most effective separation of groups was obtained with the slow component of the rate at which the dose was accumulated (Fig. 5). In severely hyperthyroid subjects, however, the distinction between rapid and slow components occasionally could not be made. The types of rate of uptake curves encountered have been illustrated in Fig. 1.

Analysis of the slow component of the rate to dose revealed that 50 per cent of the euthyroid group had rates below 2.2 per cent/hour, and 96 per cent had rates below 4.8 per cent/hour; whereas 50 per cent of the hyperthyroid group had rates above 25.2 per cent/hour, 96 per cent had rates above 8.4 per cent/hour, and 100 per cent had rates above 6.0 per cent/hour.

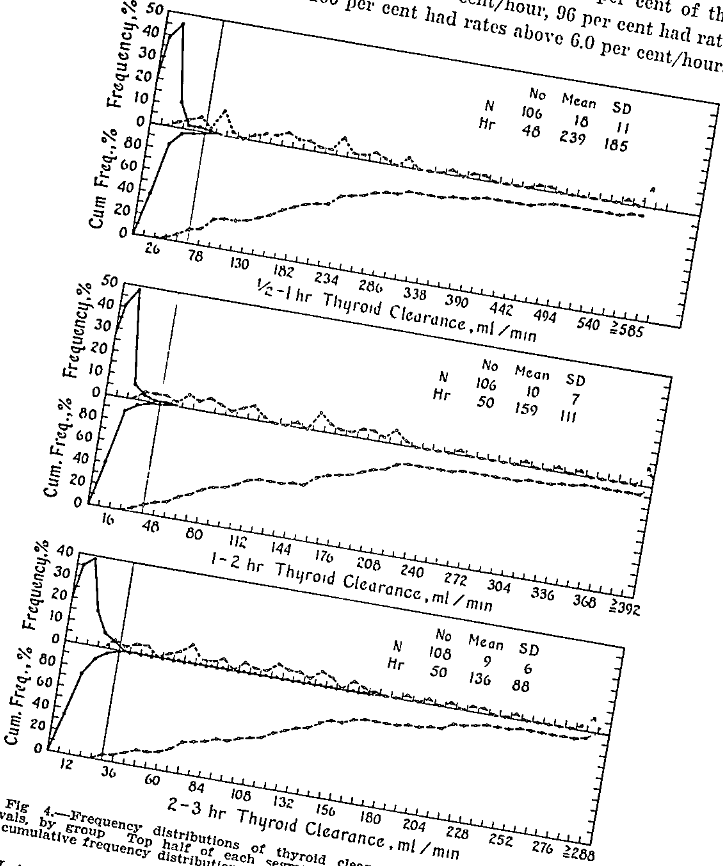

Fig 4.—Frequency distributions of thyroid clearance of radioiodine at various time intervals, by group Top half of each segment represents frequency distributions, bottom half, cumulative frequency distributions Remainder of legend as in Fig 3

Interrelationships.—Intercorrelations were calculated among the measures as shown in Table I. Scatter diagrams for the hyperthyroid patients are presented in Fig. 6. For the euthyroid as well as the hyperthyroid patients, the correlations among the measures were in general moderately high. The best correlation observed among the hyperthyroid patients was between the 3-hour thyroid uptake and the slow component of the rate to dose (0.93).

When both euthyroid and hyperthyroid patients were analyzed together, the intercorrelations rose, as would be expected, ranging in value from 0.77 for the 24-hour uptake and the 2-3 hour I^{131} clearance to 0.96 for the 3-hour uptake and the slow component of the rate to dose. The 3-hour uptake was more highly correlated with the other two measures than was the 24-hour uptake. The 3-hour uptake and the 2-3 hour I^{131} clearance, rather than values at earlier time intervals, were selected for these correlation studies, because of their slight superiority in separating the euthyroid and hyperthyroid groups. The correlation between the 3-hour uptake and the ½-1 hour thyroid I^{131} clearance in hyperthyroid patients was 0.75, slightly higher than the value of 0.64 obtained when the 2-3 hour clearance was used. The correlation between the ½-1 hour and 2-3 hour clearances was 0.70.

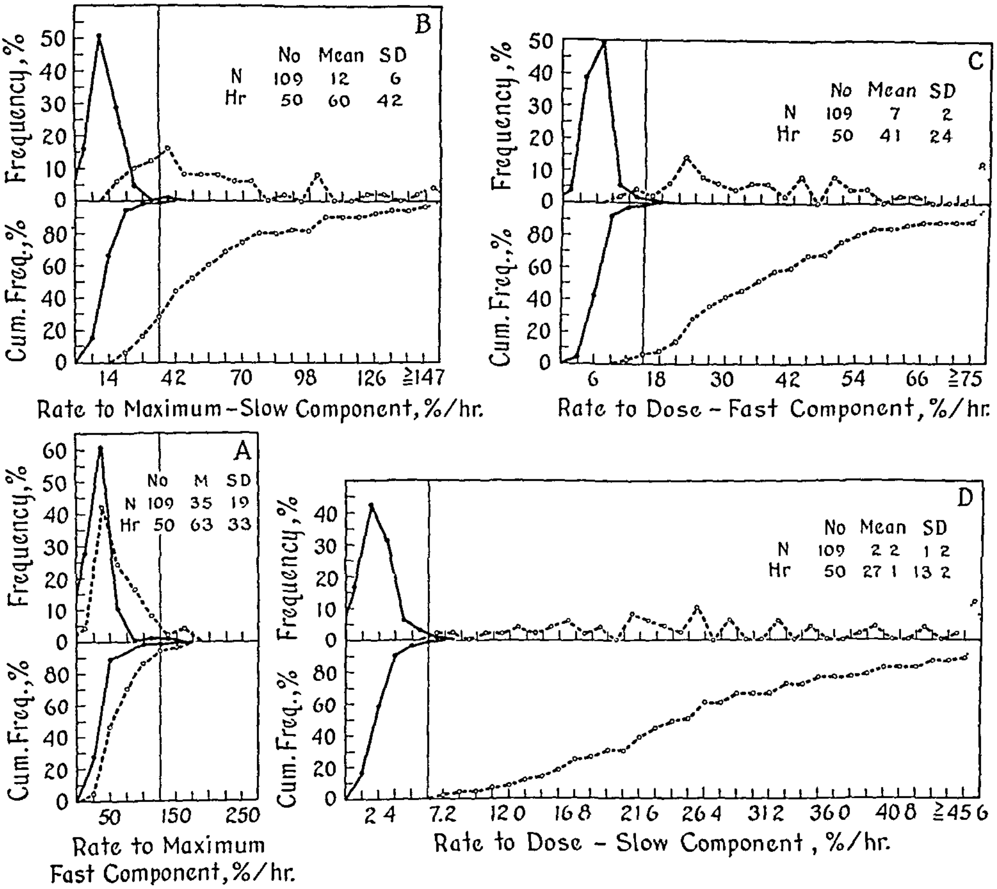

Fig 5.—Frequency distributions of rates of thyroid uptake of radioiodine, by component and group Remainder of legend as in Fig 4

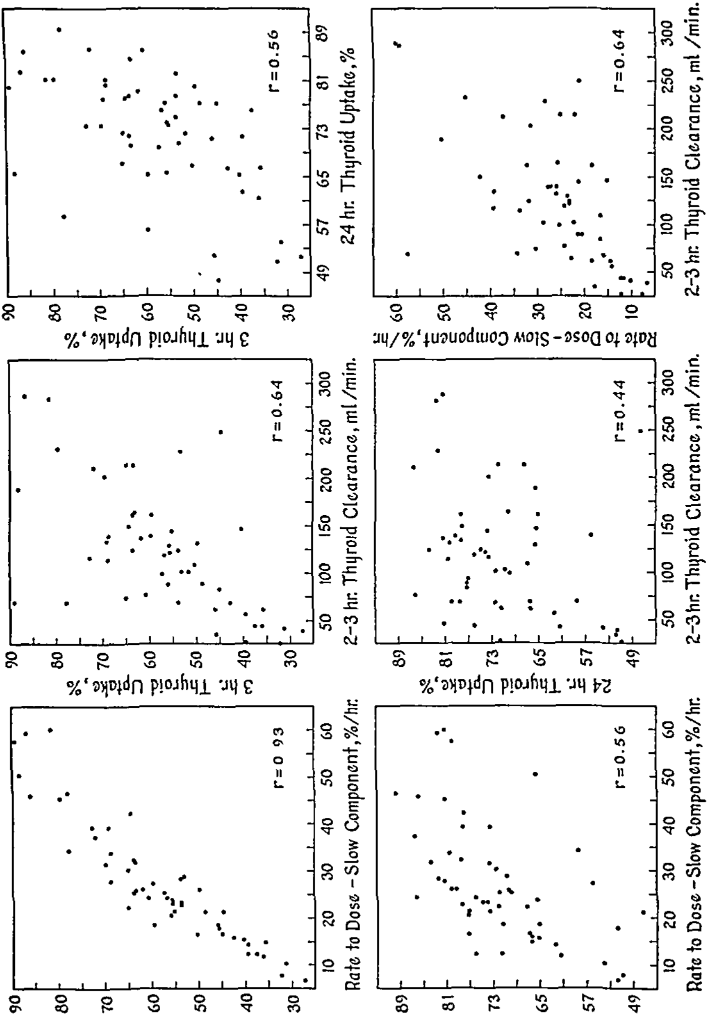

Fig 6 —Scatter diagrams and intercorrelations among tests within the hyperthyroid range

The 3-hour thyroid uptake was clearly as effective as the other measures in separating the euthyroid and hyperthyroid groups as shown in Figs. 3, 4, and 5. In view of this and the high intercorrelations of the 3-hour uptake with the rate of uptake and the thyroid I^{131} clearance, this simplest of measures is probably adequately representative of the others for practical clinical purposes. On the same bases, it is apparent that the 24-hour uptake is somewhat less effective and less representative.

TABLE I. INTERCORRELATIONS AMONG TESTS

	24 HR. TU	RTU-S	2-3 HR. TC
3 hr. TU			
Euthyroids	0.68	0.76	0.74
Hyperthyroids	0.56	0.93	0.64
Both	0.93	0.96	0.84
24 hr. TU			
Euthyroids		0.61	0.47
Hyperthyroids		0.56	0.44
Both		0.86	0.77
RTU-S			
Euthyroids			0.60
Hyperthyroids			0.64
Both			0.84

Number of cases: euthyroids 50, hyperthyroids 50, both 100.
TU = Thyroid uptake.
TC = Thyroid clearance.
RTU-S = Rate of thyroid uptake of dose, slow component.

DISCUSSION

Previous reports have shown that the thyroid I^{131} uptake will distinguish hyperthyroid from euthyroid subjects within the first hour following a tracer dose.[8-10] Separation was considered as good as that obtained with the 24-hour thyroid uptake. Results were similar whether an oral or an intravenous dose was given.[9]

We found the uptake at 3 hours to be the best uptake measurement after an oral tracer dose, although it had little advantage over the 1- and 2-hour time intervals, and there was good separation of groups as early as ½ hour after the dose. The 24-hour uptake was less effective, judging by the degree of separation of the means of the groups, the amount of overlapping, and the spread of hyperthyroid values. This is not surprising when one considers the shape of the uptake curve in hyperthyroid patients in whom, in contrast to euthyroid subjects, the major accumulation of I^{131} by the thyroid occurs within the first 8 hours. Also, in the more severely hyperthyroid patients there is a significant decline in thyroid radioactivity during the second 12-hour period as a result of the release of labeled hormone. By the same token one would expect uptake values up to 8 hours to be as discriminative as the 3-hour value.[11]

It has been claimed that thyroid clearance of I^{131} remains constant for several hours after the dose is given.[5, 6, 12] Our observations indicated a progressive decrease in the I^{131} clearance during the first 3 hours, as a rule. This was more apparent in hyperthyroid patients than in euthyroid subjects whose initial clearance values are small. Though the absolute decrease in clearance in euthyroid patients might be ignored as small, the proportionate decrease is similar, on the average, to that in hyperthyroid patients. The lack of constancy of radioiodine clearance is best studied in the hyperthyroid patient whose values are sufficiently large to reveal striking absolute changes.

In the study by Myant, Pochin, and Goldie[9] observations on 2 hyperthyroid patients revealed a progressive decrease in thyroid I^{131} clearance with time, though the authors concluded the clearance remained constant. Berson and associates[6, 12, 13] have emphasized the constancy of thyroid I^{131} clearance over periods of several hours and have utilized this assumption in calculations of the rate of thyroidal iodide binding and the rate of hormone formation. Their data consisted of (a) 4 euthyroid subjects each of whom showed a slight decrease in clearance upon comparison of the first ½ hour with the last 2½ hours of a 3 hour period,[6] and (b) 3 hyperthyroid patients each of whom showed *nonrandom* changes (first increasing then decreasing) in thyroid I^{131} clearance during the brief periods they were studied.[12] The sequence of values clearly reflected the differences in the rate of change of thyroid uptake as compared to the rate of change in plasma concentration. These data seem to us inadequate support for the conclusions drawn by the authors. They have furthermore implied that the thyroid I^{131} clearance may only be constant for very short periods after the dose because of the rapid appearance of significant concentrations of PBI^{131} in the plasma, particularly in very active glands.

TABLE II PERCENTAGE OF PLASMA RADIOACTIVITY THAT IS PROTEIN BOUND AT VARIOUS TIME INTERVALS AFTER THE DOSE IN REPRESENTATIVE HYPERTHYROID, EUTHYROID, AND HYPOTHYROID PATIENTS

		PLASMA (PBI^{131}/TOTAL I^{131}) × 100						THYROID UPTAKE	
PATIENTS	DOSE	½ HR (%)	1 HR (%)	2 HR (%)	3 HR (%)	5 HR (%)	24 HR (%)	3 HR (%)	24 HR (%)
Hyperthyroid									
RCl	Oral	0.7	1.0	0.8	1.6	—	62.9	35.6	61.0
EBl	Oral	—	1.2	1.6	3.1	9.9	86.7	53.0	70.6
ETo	Oral	—	0.9	2.8	7.7	32.5	76.2	69.8	73.5
VSp	Oral	—	2.1	5.2	11.4	37.2	81.2	63.7	71.8
SBu	I V	1.2	1.7	1.3	1.6	—	25.5	26.3	48.2
Euthyroid									
RRe	Oral	—	1.6	1.5	1.3	2.3	3.1	8.8	19.9
GWo	Oral	—	1.3	1.4	1.2	1.5	4.3	12.2	31.5
HLo	Oral	—	1.5	1.6	1.7	1.5	6.1	9.7	22.2
AMi	Oral	—	2.5	2.6	2.3	2.2	4.8	7.5	19.4
JGu	I V	1.1	1.3	1.4	1.5	—	6.0	12.5	29.3
Hypothyroid									
OJo	Oral	—	0.3	0.3	0.3	0.6	2.8	4.0	3.5
NWi	Oral	—	0.1	0.2	0.3	0.3	1.1	4.4	3.9
AWe	Oral	—	0.8	1.0	1.5	2.6	8.6	10.0	15.9
JKe	Oral	—	0.3	0.2	0.4	0.4	1.4	4.1	2.1
HSa	Oral	—	1.9	1.6	1.6	1.9	4.6	4.7	7.1

This presumably is their explanation for the rapid decrease in clearance most evident in severely hyperthyroid patients beyond the first half hour. We have tested this thesis by measuring plasma PBI^{131} concentrations ½, 1, 2, 3, 5, and 24 hours after the dose[14] and calculating conversion ratios as shown in Table II. Little difference is noted between the euthyroid and hyperthyroid patients within the first two hours. Even in severely hyperthyroid patients

(ETo and VSp) the PBI^{131} is only a small fraction of the circulating radioiodine at 3 hours and so could only have a negligible effect in the computation of the thyroid clearance. The appearance of significant amounts of labeled hormone in the blood is not as rapid as presupposed by these workers, and the concentration of labeled hormone is apparently not a significant factor in the progressive decrease in thyroid I^{131} clearance with time during the early hours after a dose.

The decreasing I^{131} clearance most likely reflects the changing distribution of radioiodine. The intravascular-extravascular distribution of a test substance influences any clearance method which uses a single dose technique, as has been discussed by Smith[15] in relation to renal clearances. An essential simplifying condition is constancy of the blood level. This does not exist after a single injection because of the rapid flow of the substance from the intravascular to the extravascular compartments, equilibrium not being reached for many hours. Though one might ignore slight losses elsewhere, as through renal excretion, one cannot ignore the significant fraction flowing into the extravascular nonthyroidal space during the early hours when a clearance calculation is feasible. For the thyroid I^{131} clearance to be constant under these conditions, the rate of change in plasma I^{131} concentration must be proportional to the rate of change in gland uptake of I^{131}. This is an unlikely event under the circumstances and is not borne out by our data.

The true thyroidal clearance of iodine (I^{127}) is probably fairly constant over a given interval of time. However, it is not reflected accurately in clearances of I^{131}, although at some one variable time after the tracer dose the true clearance may be approximated. Nevertheless, I^{131} clearance studies, such as have been presented, will distinguish hyperthyroid from euthyroid individuals and have practical diagnostic value.

Little clinical application has been made of the rate of accumulation of I^{131} by the thyroid gland. Luellen, Keating, and co-workers,[3] studying small groups, observed broad separation of hyperthyroid and euthyroid patients. Their data, like ours, showed a rapid and a slow component in the rate of uptake after an oral dose. The rapid component is probably related to absorptive factors, reflecting the rapid rise in plasma I^{131} which reaches a peak approximately one hour after a fasting oral dose, and only indirectly reflecting the thyroid functional state. The slow component represents the diminishing increments of thyroid uptake as the plasma concentration decreases gradually. It reflects the thyroid functional state directly and was more effective than the rapid component in separating the hyperthyroid and euthyroid groups. Also, the rate at which the dose was taken up during the first 3 hours was more discriminative than the rate at which the maximum uptake was approached. This is to be expected since the ultimate level of uptake achieved is taken into account as well as the actual rate of uptake.

The rate of thyroid uptake was at least as effective as the thyroid I^{131} clearance in differentiating hyperthyroid from euthyroid patients. However, neither of these measurements was any more discriminative than the simpler 3-hour uptake. All three measures reflect the iodine-accumulating function of the thyroid gland, and their intercorrelations were in fact high when the measurements were made simultaneously in the same patients In view of these observations the 3 hour uptake as the simplest measure becomes the clinical procedure of choice, and little additional information is gained by adding the other two tests

SUMMARY

Tests were performed simultaneously in 109 euthyroid and 50 hyperthyroid patients *Plasma thyroid clearances were obtained* ½ 1, 1 2, and 2 3 hours after an oral dose of I^{131} A progressive decrease in I^{131} clearance was the rule Clearances at each time interval were of equal value in separating hyperthyroid from euthyroid patients The rates at which the dose was taken up and the maximum reached were also calculated Two components, the first fast, the second slower, were evident in the rate curves The slow components were more discriminative than the fast, and the rate to dose was more discriminative than the rate to maximum Uptakes at ½, 1, 2, and 3 hours each separated the groups effectively, the 2 and 3 hour measurements being best and equivalent in value to the clearance and the dose rate The 24 hour uptake was less discriminative The 3 hour uptake, rate to dose, and thyroid clearance were highly interdependent Correlations among these three measurements were first and second 0 96, first and third 0 85, and second and third 0 84 The 3 hour uptake was representative of and as effective as the more complicated measures

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THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

V. The Effect of Supplementary Sulfur-Containing Dietary Amino Acids Upon the Die-Away Plots of Autosynthesized Albumins and Gamma Globulins Labeled With S^{35}

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S. Howard Armstrong, Jr., M.D.
Chicago, Ill.

INTRODUCTION

Investigation of the apparent turnover times of plasma protein fractions autosynthesized following oral S^{35} has failed to develop, either in our studies[1-3] or in those of Volwiler and his associates,[4] a method of measuring protein degradation rates acceptable for extensive clinical investigation. Autosynthesized protein fractions have usually shown longer apparent turnover times than have comparable intravenously injected in vitro I^{131} or in vivo S^{35}-labeled fractions. These discrepancies have often been attributed to reutilization (recycling) of the isotope tag following its liberation during catabolism of labeled body proteins.[5-7] Luck recently has written: "It is common practice to minimize this source of error by the administration of relatively large quantities of unlabeled cysteine, methionine, or such other amino acids, which by entry into the amino acid pool will dilute out the labeled amino acid released from the protein during metabolism. How effective this device is cannot be determined."[8]

In this study, we have (a) investigated the effect of feeding unlabeled sulfur containing amino acid supplements upon the die-away plots of autosynthesized S^{35}-labeled protein fractions; (b) appraised the suggestion of Volwiler's group[4] that cysteine, since it enters less into the total body organic sulfur pool than methionine, will thus be less subject to reutilization; and (c) compared the intravenous to the oral route for the administration of labeled amino acids.

MATERIALS AND METHODS

Choice of Subjects.—A list of subjects, their diagnoses, and their plasma protein levels are given in Table I.

From the Hektoen Institute of Medical Research of the Cook County Hospital and the Departments of Medicine of the University of Illinois College of Medicine and Northwestern University Medical School.

Presented in part April 19, 1956, at a symposium in Atlantic City sponsored by the Metabolism and Nutrition Study Section of the National Institutes of Health.

These studies have been supported by Grant H-1511 (C, C2) from the National Heart Institute of the United States Public Health Service.

We are indebted to Warden Fred Hertwig of the Cook County Hospital and to the Drs. Leonard H. and Louis D. Weissman Medical Research Foundation for the provision of basic facilities for protein research at the Hektoen Institute.

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Two subjects (J. M. and R. S.) were young adult men with no clinical evidence of abnormal protein metabolism.

Seven additional subjects were chosen to exemplify a range of hypoalbuminemia and represent a variety of nutritional problems. In three, the hypoalbuminemia was severe (T. C., W. S., and T. N.); the remainder showed serum albumin levels ranging up to 3.3 Gm. per 100 c.c.

TABLE I*

PATIENT	DIAGNOSIS	SERUM PROTEINS (GM./100 C.C.) TOTAL	ALBUMINS	GLOBULINS
T. N.	Chronic pancreatitis (autopsy)	4.0	0.7	3.3
Al. J.	Surgical pancreatectomy	7.1	2.8	4.3
Au. J.	Chronic calcific pancreatitis	6.5	3.4	3.1
M. L.	Chronic calcific pancreatitis	6.2	2.9	3.3
L. D.	Idiopathic adult sprue	5.5	2.4	3.1
W. S.	[illegible] (defec [illegible]	3.0	0.8	2.2
T. C.	[illegible] pos [illegible]	3.7	1.3	2.4
J. M.	Nutritionally normal (cerebral palsy)	7.8	3.9	3.9
R. S.	Nutritionally normal (transverse myelitis)	7.9	4.0	3.9

*A/G ratio by paper electrophoresis as described in reference 1.

TABLE II*

PATIENT	LABEL ROUTE	DOSE (MC.)	TYPE	ORAL SUPPLEMENT DOSE	TIMING AFTER LABEL ADMINISTRATION	FIGURE IN THIS PAPER
T. N.	Oral	0.5	S^{35} methionine	None		1
Al. J.	Oral	0.5	S^{35} methionine	None		1
Au. J.	Oral	0.5	S^{35} methionine	None		1
M. L.	Oral	0.5	S^{35} methionine	None		1
L. D.	Oral	0.5	S^{35} methionine	None		1
W. S. a	Oral	0.5	S^{35} methionine	None		2
b	Oral	0.5	S^{35} methionine	None		2
c	I.V.	0.1	I^{131} albumin	Lugol's solution		2
d	I.V.	0.1	I^{131} α globulins	Lugol's solution		2
T. C. a	Oral	0.5	S^{35} L-cysteine	5 Gm. cysteine 5 Gm. methionine	Day 108 to day 124	3 and 5
b	I.V.	0.1	I^{131} albumin	Lugol's solution		3
c	I.V.	0.1	I^{131} α globulins	Lugol's solution		3
J. M. a	Oral	3.0†	S^{35} yeast	5 Gm. cysteine 5 Gm. methionine	Day 275 to day 292	4
b	Oral	0.5	S^{35} cysteine	Same	Day 18 to day 42	4
c	I.V.	0.5	S^{35} cysteine	Same	Day 0 to day 21	6
d	I.V.	0.5	S^{35} cysteine	Same	Day 0 to day 14	8
R. S. a	I.V.	0.5	S^{35} cysteine	5 Gm. cysteine 5 Gm. methionine	Day 0 to day 14	7
b	I.V.	0.5	S^{35} cysteine	Same	Same	9

*Protein fractions labeled with I^{131} for intravenous injection were obtained through Dr. Donalee Tabern of Abbott Laboratories, North Chicago, Illinois. These fractions were not especially iodinated for this metabolic study.

†The reasons for the high dosage are given, for this special instance, in reference 3.

Administration of Labels and Supplements; Radioactivity Measurements.—A list of the labeling and supplementation experiments, detailing label, route, and timing, is given for these subjects in Table II, together with a list of corresponding data plots by figure number.

Except when noted in legends, oral and intravenous S^{35} dosage level approximated 0.5 mc.

Supplementary sulfur-containing dietary amino acids were administered orally as methionine and cysteine, 5 Gm. each, daily in divided doses for periods of 14 to 21 days. This is, in our experience, the maximum dose which can be tolerated without producing loathing or gastrointestinal disturbances. Subjects were maintained on the regular hospital diet without the controls usual on a metabolic ward.

Serial sera were withdrawn for periods up to 50 days. In the two instances, where the studies were carried out 275 and 110 days after labeling, residual radioactivity was too low to permit measurement of electrophoretically separated protein fractions, and, therefore, unfractionated serum protein samples were substituted. In all other studies, methods of fractionation, extraction, measurement of component concentrations, and radioactivity were carried out as previously described,[1] and die-away plots were made of the resultant data in the customary semilogarithmic fashion against time.

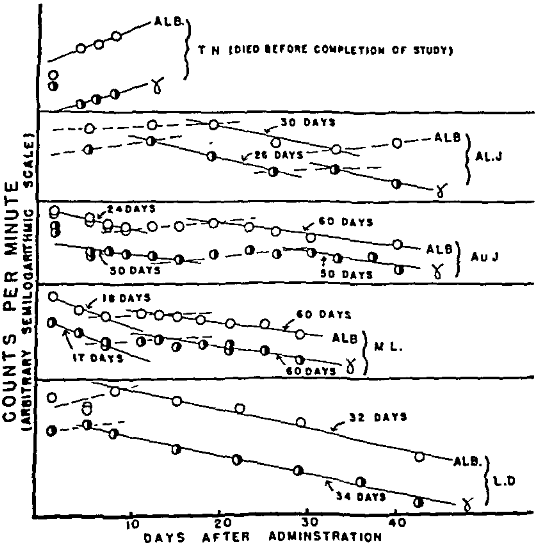

Fig. 1.—Albumin and gamma globulin die-away plots in 5 patients with abnormal gastrointestinal tracts following methionine S^{35} ingestion (see Tables I and II).

Apparent turnover times corresponding to slopes of selected segments are indicated next to the arrows.

RESULTS

Die-Away Plots Following Oral S^{35} Methionine Without Unlabeled S Amino Acid Supplementation.—Customarily, the slope of the first 5 days of a die-away plot is considered chiefly representative of equilibration of the intravascular labeled plasma protein with the extravascular pool. The slope for the next 10 days or so is considered chiefly characteristic of the rate of metabolic degradation of the labeled protein.

In our previously published studies, we have reported a very frequent flattening of the slope of the die-away plots which may begin late in this "metabolic period" and, particularly following oral S^{35}-labeled yeast, may become quite striking between the twentieth and fortieth days. More rarely have we noticed such flattening with an intravenously injected in vitro labeled protein.

In Fig. 1 are presented albumin and gamma globulin die-away plots of the first 5 subjects of Tables I and II. Diagnostic classification is less important here than the form of the die away plots, particularly in the first 5 days representative of "equilibration" when the slope should, in the absence of isotope reutilization, be sharply downward The two top and the bottom subjects have *rising* specific activity levels for albumins and gamma globulins during these first days The two middle patients in Fig 1 have specific activity levels which, although showing an initial decline in the mixing and metabolic periods, give *some evidence of a rising level before a final falling slope* Whereas the top two patients could not be described as clinically in the "steady state," the bottom three could so be described Therefore, these roller coaster die away plots clearly preclude any graphic translation of slopes into apparent turnover times The initially encountered rising slopes suggest a label recycling so great as to obliterate completely the downward slope ordinarily characteristic of the equilibration period We thus concur in the suggestion of Volwiler's group that the oral S^{3} methionine label is not adaptable to clinical studies of plasma protein fraction turnover by die away plot

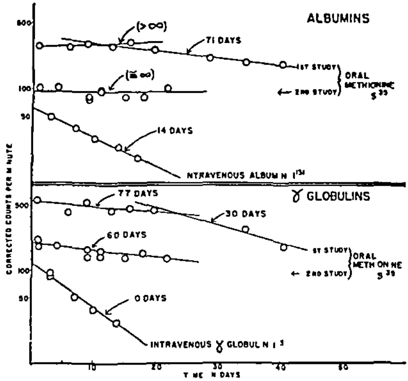

Fig 2—Patient W S Comparison of the die away plots of albumins and gamma globulins following oral S^{35} methionine and following intravenous I^{131} labeled protein fractions

The top two lines in the upper and lower diagrams represent duplicate studies with the oral isotope and show in the first study terminal dropping off rather than a terminal flattening of the die away plot A glance shows the great difference between the I^{131} and S^{35} slopes in this subject Corresponding apparent turnover times are indicated as in Fig 1

Comparison in the Same Hypoalbuminemic Subject of the S^{35} Methionine Oral Label With the I^{131} Intravenous Label of Albumins and Gamma Globulins—Fig 2 presents three sequential studies of subject W S (Tables I and II) It will be noted that in both studies following oral labeling with S^{35} methionine performed without use of supplementation (a) the albumin die away plots showed either unchanging or increasing specific activity for almost 3 weeks, and (b) the companion die away plots for gamma globulins while not rising or flat, show a very slow decrease with time In contrast, the slope of the plots following intravenous I^{131} labeled proteins showed a steep decline This corresponds for albumins to an apparent turnover time 3 or 4 days shorter than is usually observed in normal subjects. The apparent turnover time for gamma globulins is also somewhat shorter than in the normal. The findings in this subject represent the most accentuated discrepancy between the I^{131} and S^{35} label that we have encountered and confirms our dissatisfaction with oral methionine as a valid label for clinical investigation.

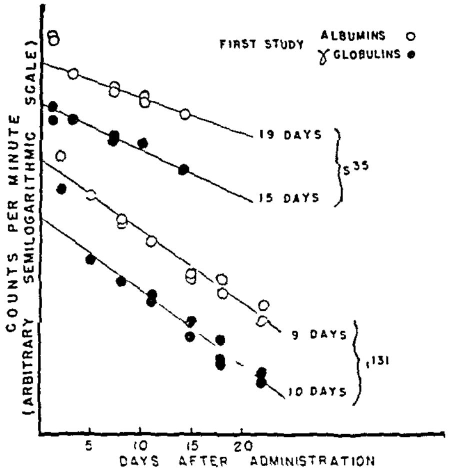

Fig. 3.—Patient T. C. Oral L-cysteine and I.V. I^{131}. Die-away plots on a patient with nutritional hypoalbuminemia secondary to prolonged low protein intake.

The upper two plots are for albumins and gamma globulins following oral S^{35} L-cysteine. The lower two plots are following the intravenous injection of I^{131}-labeled fractions.

Apparent turnover times corresponding to the slopes during the "metabolic decay period" between day 5 and day 14 are indicated to the right of the plots.

Comparison in a Hypoalbuminemic Subject of the S^{35} Cysteine Oral Label With the I^{131} Intravenous Label for Albumins and Gamma Globulins.—Fig. 3 illustrates another nutritional hypoalbuminemic patient (T. C.); here is a comparison between I^{131} and S^{35} apparent turnover times where the oral label was S^{35} L-cysteine and where supplementation was not employed. Here the slope, during the metabolic period, yields apparent turnover times which are significantly shorter than are usually found after the oral S^{35} methionine label, but are still considerably longer than with comparable findings with the I^{131} intravenous label.

This result substantiates the suggestion of Volwiler and his associates that cysteine may be preferable as a labeling agent to methionine from a recycling standpoint. But while lessening, cysteine clearly does not eliminate the discrepancy between the apparent turnover time obtained by the S^{35} and I^{131} method in the same subject.

Comparison of the Effect of Dietary Unlabeled S Amino Acid Supplements on the Apparent Turnover Times of Plasma Proteins at Various Intervals After Oral and Intravenous Administration of the S^{35} Label.—

Unfractionated serum proteins months after oral labeling with S^{35} methionine: Supplementary dietary sulfur containing amino acids were fed to one normal (Fig. 4, top) and one hypoproteinemic (Fig. 5) patient, 275 and 110 days, respectively, after the oral S^{35} label. In each patient, there occurred an immediate steepening of the slope corresponding to an apparent turnover time of 17 days. Compared to the mean of the apparent turnover time of albumins and gamma globulins as measured by intravenously injected I^{131}-labeled fractions, this apparent turnover time was 4 days longer for the normal patient (J. M.) and 7½ days longer for the hypoproteinemic patient (T. C). In the same manner, the mean apparent turnover time after oral S^{35} L-cysteine in the hypoproteinemic patient was also 17 days. Thus, while supplementary feedings secured a marked increase in apparent turnover time, a discrepancy between the S^{35} and intravenous I^{131} label is still apparent.

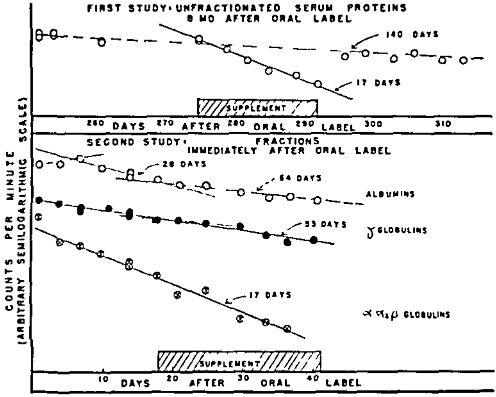

Fig 4 —Patient J M Comparison of effect on die-away plots of oral unlabeled S amino acid supplementation started 275 days (top section) and 18 days (bottom section) after the oral dose of S^{35} amino acid

Because of low specific radioactivities, fractionation in the first study (upper) was impractical. Fractions prepared as described elsewhere are plotted for the second (lower) study

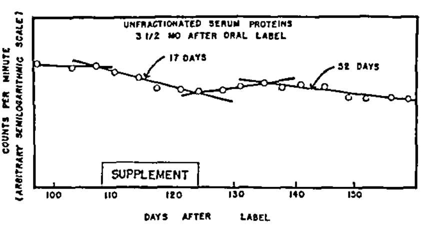

Fig 5 —Patient T C. Effect on die-away plot of oral unlabeled S amino acid supplementation started 110 days after oral dose of S^{35} amino acid in hypoproteinemic subject

Because of low specific radioactivities, fractionation was impractical

Upon discontinuation of the supplement, the die-away plot for both patients took a sharp upward swing, and then resumed the previous slow steady decline, so that a superimposed straight edge connects the first and last portions of the plot.

Fractionated serum proteins eighteen days after oral labeling with S^{35} methionine: In a second study in Patient J. M., the supplement was begun 18 days after oral labeling. The findings (Fig. 4, bottom) are quite different.

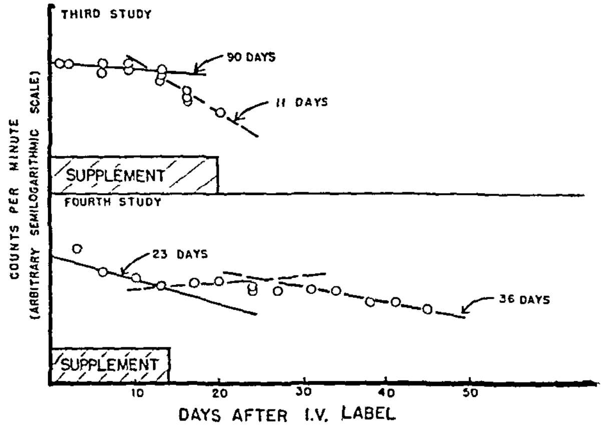

Fig. 6.—Patient J. M. Effect of immediate oral supplementation (5 Gm. cysteine, 5 Gm. methionine per day) on albumin die-away plots following intravenous S^{35} cysteine (same metabolically normal subject as in Fig. 4).

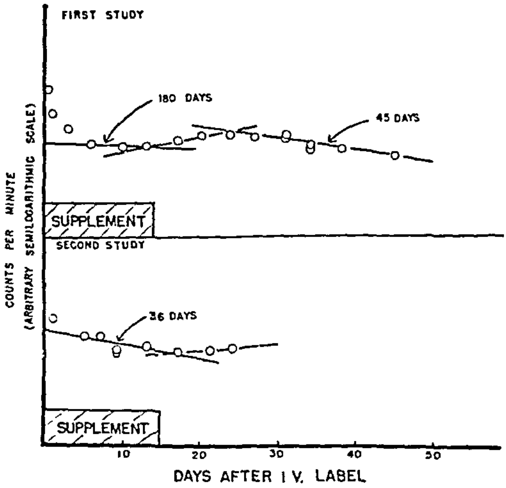

Fig. 7.—Patient R. S. Effect of immediate oral supplementation (5 Gm. cysteine, 5 Gm. methionine per day) on albumin die-away plots following intravenous S^{35} cysteine in a second metabolically normal subject.

The initial upward albumins slope and flat gamma globulins slope, prior to the administration of the supplement, suggests intensive isotope reutilization during this period. More important are these findings: (1) the albumins slope turns downward *before* the supplement is started; (2) the slope flattens rather than steepens during the supplement administration; and (3) neither the albumins nor the gamma globulins slope becomes as steep as the slope for unfractionated plasma proteins (Fig. 4, top) when the supplement is given 275 days after the oral label.

Following the analogy of Schoenheimer and his group's classic studies on N^{15},[9] these findings suggest that only after the oral label has become well distributed through the body's tissue protein stores (whose turnover rates are very slow) can recycling be repressed by oral unlabeled supplements Such distribution takes months, not days When, after but two weeks or so, such organs as the liver have specific activities about as high as those of simultaneously obtained plasma proteins,[2] oral supplements at the dosage used seem ineffective

Albumins immediately after intravenous labeling with S^{35} cysteine The above results suggested that failure to minimize recycling might still be due to several possibilities, namely, (1) the oral supplement was too small (2) it was given too late after the initial oral label, and (3) that the intravenous route (by initial partial liver circumvention) might be preferable to the oral route for labeling

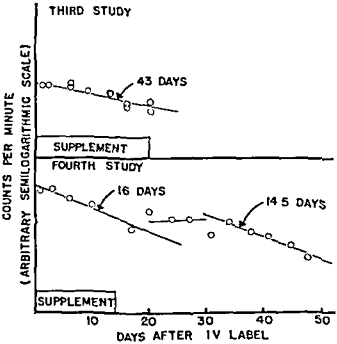

Fig 8

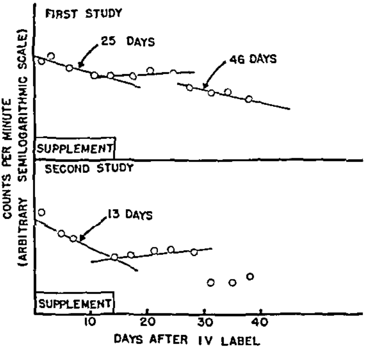

Fig 9

Fig 8 —Patient J M Effect of immediate oral supplementation of gamma globulin die away plots following intravenous S^3 cysteine (Compare to simultaneous study of albumins in the same subject Fig 6

Fig 9 —Patient R S Effect of immediate oral supplementation on gamma globulin die away plots following intravenous S^{35} cysteine (Compare to simultaneous study of albumins in the same subject Fig 7

Figs 6 and 7 present duplicate studies in the two normal patients for the albumin die away plots when the supplement was given immediately following intravenous labeling with S^3 cysteine It will be noted that (1) there is no consistency in comparison of the apparent turnover times or the shapes of the die away plots, either between the two patients or between duplicate studies *in the same patient, although their clinical status during the study remained* unchanged, (2) the shortest apparent turnover time, 23 days in Patient J M (Fig 6, bottom) was still six days longer than the figure obtained for albumins with the intravenous I^{131} label, (3) when sufficient residual radioactivity remained to permit extended observation, a rise in the slope followed by a gradual decrement occurred when the supplement was discontinued The resultant roller coaster appearance of the die away plots is illustrated in Figs 6 (bottom), 7 (top) and 8 (bottom); (4) the apparent turnover time characteristic of the terminal portion of the die-away plot in the fourth study of Patient J. M. (Fig. 6, bottom) was somewhat longer than during the second week after labeling. The reverse of this relationship is seen in the first study on R. S. (Fig. 7, top).

Gamma globulins immediately after intravenous labeling with S^{35} cysteine: Figs. 8 and 9 illustrate the gamma globulin die-away plots obtained simultaneously with the above albumin studies. The same remarks pertaining to the albumins are applicable here.

TABLE III. APPARENT TURNOVER TIMES OF ALBUMINS AND GLOBULINS IN A NORMAL PATIENT (J. M.) OBTAINED BY DIFFERENT LABELING PROCEDURES

METHODS OF LABELING	PERIOD STUDIED	APPARENT TURNOVER TIME (DAYS)	
		ALBUMINS	GAMMA GLOBULINS
S^{35} oral yeast, no supplement	7th-40th day	64	40
S^{35} oral cysteine, no supplement	7th-18th day	28	41
S^{35} oral cysteine, with supplement	18th-40th day	64	53
I^{131} intravenous fractions, Lugol's supplement	1st-20th day	17	12

DISCUSSION

The dilemma in the interpretation is well illustrated by Table III, in which apparent turnover times for the albumins and gamma globulins of a single subject, as measured by various labeling and supplement schedules, are summarized.

The discouraging implications for a valid method to determine turnover times of autosynthesized plasma proteins in clinical investigation clearly are not limited to the S^{35} level alone; the C^{14} and the N^{15} labels, to a greater or lesser degree, probably lead to the same methodological morass.

That the use of unlabeled amino acid supplements has failed, in the experiments described in this paper, to influence this unfavorable picture is perhaps not surprising from the recent animal work of Loftfield and Harris.[10] In their important paper, which summarizes and adds new evidence for the view that protein synthesis occurs directly from an intracellular free amino acid pool, they also bring evidence that in the rat liver, at least, this pool does not enjoy immediate equilibration with circulating free plasma amino acids, L-valine C^{14}, L-isoleucine C^{14}, and L-leucine C^{14} were used. Working with the responses of the ferritin synthesis system to iron stimulation, they failed to demonstrate any influence of massive intravenous injection of *unlabeled* amino acids on the specific radioactivity of the newly synthesized ferritin.

SUMMARY AND CONCLUSIONS

In this study, we have extended our previously reported comparisons, in the same subjects, of apparent turnover times of serum albumin and gamma globulin, autosynthesized following oral S^{35} ingestion, or intravenously injected following in vitro I^{131} or in vivo S^{35} labeling to two new situations: (1)

the intravenous injection of the labeling amino acid, and (2) the administration of large amounts of unlabeled S amino acids at various periods during the die away plot in an effort to minimize isotope reutilization or "recycling"

The use of S^{35} cysteine, whether given orally or intravenously, whether or not accompanied by oral supplements of unlabeled S containing amino acids in dosage as high as 10 Gm per day, has provided die away plots for albumins and gamma globulins whose slopes do *not* have sufficient consistency or reproducibility to permit valid metabolic interpretation in terms of apparent turnover times for serum albumins or gamma globulins Even with immediate unlabeled supplement, the results are only *slightly* more satisfactory than following the oral S^{35} methionine label The discrepancies in apparent turnover times between autosynthesized and in vitro or in vivo label intravenously injected fractions have *not* been eliminated

While an apparent effect of oral supplementation has been noted on the slope of the die away plots some *months* after the oral S^{35} amino acid label, a method based on this tenuous finding would be hardly of value for clinical investigation As a rule, one wants turnover data in the immediate future and not after months in which unpredictable clinical phenomena can occur

In our opinion, these results make it very unlikely that oral isotope administration, except in the most crude empirical fashion, is going to prove a valid tool for the study of protein turnover in disease states

We are grateful to Miss G Robey for technical assistance and to Miss J Peters of the Neurological Service at Cook County Hospital for metabolic nursing assistance

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THE SIGNIFICANCE OF INTERACTIONS BETWEEN PROTEINS AND LIPIDS FOR SEROLOGIC CANCER DIAGNOSIS

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IN A previous study[1] the values of 2 protein fractions were measured on 578 sera obtained from the Chicago Lying-in Hospital. The serum proteins were precipitated onto strips of filter paper and extracted with 38.5 and 31 (w/v) per cent ammonium sulfate solutions. The first fraction, considered to be a more soluble albumin, was designated Fract. S; the proteins extracted with the 31 per cent solution approximate the total albumin. It was observed that at the same total albumin level the average Fract. S values were significantly lower in sera of patients with active malignancies (called cancer sera) than in sera of patients with nonmalignant diseases (called noncancer sera). See also Table VI of this paper. Some of the individual Fract. S values showed overlapping between the 2 groups of sera.

These experiments have now been extended. A study was made of factors which affect the magnitude of the Fract. S values, and which cause the differences between cancer and noncancer sera. Purified albumin solutions prepared from both groups of sera do not differ significantly in their Fract. S values. Addition to serum in vitro of globulin or certain steroids causes lowering of the Fract. S values, while addition of fatty acids with 6 to 12 carbon atoms has an elevating effect (Table I). Most of the lipids extracted from sera have no significant effect; however, a lipid fraction extracted from cancer sera, which is soluble in ether, acetone, and cold methanol, regularly causes lowering of the Fract. S values.

Previously we had concluded that cancer sera have a characteristic deficiency of the more soluble albumin fraction.[2-4] The results of the experiments which are summarized in Table I indicate that the differences between cancer and noncancer sera are based not on variations in the quantity or nature of the serum proteins per se, but on differences in the interactions between proteins and certain nonprotein substances, especially lipids.

In this study, centrifugation was used for separating dissolved from solid protein fractions instead of paper extraction employed in the previous study.[1]

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Some of the more recent studies were carried out by one of us (K.) in the Department of Clinical Science, University of Illinois College of Medicine, Chicago.

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The serum proteins were precipitated at a concentration of 42 6 per cent ammonium sulfate and, after centrifugation, were extracted successively with three more dilute ammonium sulfate solutions, so as to determine two albumin* fractions and globulin

TABLE I ALTERING EFFECTS OF SEVERAL SUBSTANCES ON FRACT S VALUES*

ADDED SUBSTANCE†	NUMBER OF EXPERIMENTS	CONCENTRATION PER ML SERUM	AVERAGE EFFECT PER CENT‡
Human serum globulins	8	15	–30
Cholic acid	2	2	–33
Glycodesoxycholic acid	8	2	–42
Dehydrocholic acid	4	1	–49
Progesterone	2	1	–25
Testosterone	2	1	–36
Dehydroepiandrosterone	6	1	–53
Diethylstilbestrol	2	0 3	–24
Sodium caprylate	10	1	+30
Sodium laurate	10	2	+104
Lipids§ from cancer sera	23	2	–18
from noncancer sera	25	2	–7

No altering effects were observed after addition of most inorganic salts (which do not precipitate proteins) sugars amino acids nucleic acids B vitamins calciferol cholesterol lithocholic acid cortisone estradiol and methylcholanthrene

*Fract S is the more readily extractable albumin fraction by the paper extraction method[1]

†Serum was added to a weighed amount of the substance and stirred with a glass rod until complete solution or homogenous distribution was achieved

‡The altering effects were calculated as

$$100\left(\frac{\text{Fract S of modified serum}}{\text{Fract S of unmodified serum}} - 1\right)$$

The magnitude of the effects varied in different sera The averages are given

§These lipids were soluble in acetone and cold methanol For details see text

METHODS AND MATERIALS

Most of the sera used in this series were obtained from the Department of Surgery of the University of Chicago Clinics All diagnoses of malignancies were confirmed histologically The blood was drawn in the postabsorptive stage The sera could be stored in the refrigerator for one week Hemolysis and freezing were avoided

As described previously,[1] the ammonium sulfate solutions were buffered with 0 07 M ammonium phosphate The percentage number indicates grams of $(NH_4)_2SO_4$ in 100 ml of solution

Procedure—All estimations were carried out in duplicate at 5° C The mixtures of sera (or protein solutions) and ammonium sulfate solutions were stirred with a glass rod until a homogeneous distribution of the precipitates was achieved While standing, the tubes were stoppered tightly with rubber stoppers Before centrifugation the sediment was redistributed by whirling We used a Servall centrifuge at 12,000 r p m for 20 minutes It is advisable to include one normal serum in each series in order to certify the consistency of experimental conditions

A Measure 0 5 ml serum (plasma or protein solution) into a 15 ml plastic tube, add 4 ml of 48 per cent $(NH_4)_2SO_4$ solution pH 7 3, mix, let stand for one hour, and centrifuge but do not decant Add 1 ml of a 18 1 per cent $(NH_4)_2SO_4$ solution pH 7 3, which brings the $(NH_4)_2SO_4$ concentration of the mixture to 38 2 per cent Stir and let stand for 18 to 20 hours Centrifuge and decant the supernatant into a glass tube Mix 4 ml of the clear supernatant* with 11 ml. of a 31 per cent $(NH_4)_2SO_4$ solution at pH 6.8 in a 20 ml. test tube. Heat the mixture for 3 minutes in a boiling water bath, and, after cooling, read the turbidity in a photoelectric colorimeter with a 420 mμ filter.

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*Originally we separated the albumin into 4 different fractions Evaluation of the clinical data indicated that the first albumin fraction has primary significance for cancer diagnosis Control experiments on the same sera showed that the sum of the 4 albumin fractions estimated with the more extensive method agreed with the sum of the 2 albumin fractions estimated with the described method within the limits of experimental error

B. Add to precipitate A, 10 ml. of a 31 per cent $(NH_4)_2SO_4$ solution pH 6.8 and stir. Measure 1 ml. of this mixture into a 50 ml. plastic tube, mix with 14 ml. of 31 per cent $(NH_4)_2SO_4$ solution pH 6.8, and stir. Let stand for at least 2 hours. Centrifuge, decant the supernatant into a 20 ml. test tube, heat, and read the turbidity as in A.

C. Add to the precipitate B in the 50 ml. plastic tube 15 ml. of 12 per cent $(NH_4)_2SO_4$ solution pH 6.8, stir, and let stand for at least one hour. Centrifuge, decant the supernatant into a 20 ml. test tube, heat, and read the turbidity as above.

The photoelectric colorimeter was calibrated with purified albumin solutions for extracts A and B and with globulin solutions for extracts C. The protein concentrations were calculated as grams per 100 ml. of serum or original protein solution. The calculated values from extract A were designated as Fract. A. The sum of the values of extracts A and B approximates the total albumin and was designated as TA. The values from extracts C represent the globulins, and were designated as GL. Any fibrinogen which may have been present remained in the precipitate C and was discarded.

On the average, 100 ml. of normal serum contained 0.49 Gm. of Fract. A or 11 per cent of TA. The corresponding Fract. S values were 1.12 Gm. or 25 per cent of TA.[1]

The effect of serum-lipid fractions on Fract. A values was measured by the following procedure. The sera were extracted with a 3 to 1 ethanol-ether mixture. After centrifugation the supernatant was evaporated in vacuo and the residue extracted with petroleum ether. The residue from the evaporation of the petroleum ether extract was extracted at -3° C. with acetone. After centrifugation the supernatant was evaporated in vacuo. An aliquot of the residue, corresponding to 2 ml. of the extracted serum, was mixed with 2 ml. of a test serum by stirring with a glass rod. After standing overnight in the cold, the mixture was centrifuged vigorously so that an almost clear middle layer was obtained, which was used for the analysis of Fract. A. Many of the supernatants A showed slight turbidities; they were decanted and clarified by an additional centrifugation before they were measured for the protein estimation.*

RESULTS

The ratios Fract. A/TA increase in sera as TA is decreased by dilution, but the Fract. A values show little or no change (Table II) at a dilution of the serum of 1:2. Increase of GL concentration decreases the Fract. A values. This decrease seems to be proportional to the increase of the ratios GL/TA, and the products Fract. A $\frac{TA + GL}{TA}$ vary insignificantly for different GL concentrations (Table II). Some of the substances which altered Fract. S (Table I) were tested with the centrifugation method. Comparison of Tables I and II shows that substances tested by both methods alter Fract. S and Fract. A in the same direction.

The data in Table III show a certain parallelism between the value of the products Fract. A $\frac{TA + GL}{TA}$ of sera and the magnitude of the lowering effect of the lipid fraction which was extracted from those sera. This indicates that at least a part of the variations in the values of these products is caused by interactions of proteins and lipids. Comparison of Fract. A values of purified albumin solutions (from which the lipids have been removed) prepared from cancer and from noncancer sera revealed no significant differences.

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*More recent studies have shown that the accuracy of the method is increased and its applicability extended to very turbid sera (which had to be excluded from this study) when all sera are clarified routinely by centrifugation at high speed before the analysis and when the decanted supernatants A are centrifuged again before the protein estimation.

TABLE II. EFFECTS OF IN VITRO MODIFICATIONS OF SERA OR HUMAN ALBUMIN SOLUTIONS ON THEIR FRACT. A VALUES*

	FRACT. A*	TA*	GL*	CHANGE IN FRACT. A	FRACT. A $\frac{TA + GL}{TA}$
	(GM. IN 100 ML.)			PER CENT	(GM. IN 100 ML.)
I. Dilution					
Serum	0.50	4.68	2.82		0.80
Serum + equal volume of water	0.50	2.33	1.40		0.80
II. Addition of pseudoglobulin†					
A. Serum albumin solution	0.39	3.21	0.00		0.39
B. Albumin pseudoglobulin mixture	0.25	3.23	1.63	−36	0.38
C. Equal parts of A and B	0.32	3.32	0.74	−18	0.39
D. One part of B and 3 parts of A	0.36	3.32	0.40	−8	0.39
III. Addition of nonprotein substances					
Serum alone	0.47	4.16	4.50		0.98
Serum plus 1.3 mg. of sodium caprylate per ml.	0.85‡	4.31	4.16	+81	1.67
Serum plus 0.7 mg. of dehydrocholic acid per ml.	0.10	4.09	4.50	−78	0.21
Serum plus 0.2 mg. of dehydroepiandrosterone per ml.	0.33	4.19	4.34	−30	0.66
IV. Addition of serum lipids					
Serum alone	0.37	4.20	3.10		0.64
+ lipids§ from a noncancer serum	0.35	4.20	3.10	−5	0.61
+ lipids§ from a cancer serum	0.31	4.20	3.10	−16	0.54

*Fract. A is the more readily extractable albumin by the centrifugation method described in the text. TA means total albumin, GL globulin.

†Albumin and pseudoglobulin were prepared from pooled human sera by fractionation with ammonium sulfate and dialysis.

‡In this case the supernatant was turbid.

§These lipid fractions were soluble in petroleum ether and cold acetone. For details see text.

TABLE III. COMPARISON OF CORRECTED FRACT. A* VALUES OF SEVERAL GROUPS OF SERA AND FRACT. A LOWERING EFFECTS OF THEIR LIPIDS†

SERA FROM PATIENTS	FRACT. A $\frac{TA + GL^*}{TA}$ (GM. PER 100 ML.)		CHANGE OF FRACT. A VALUES AFTER MIXING A NORMAL SERUM WITH LIPIDS† FROM THESE SERA (PER CENT)	
	RANGE	AVERAGE	RANGE	AVERAGE
3 without malignancies	0.72 to 0.61	0.66	+3 to −10	−4
4 with treated malignancies	0.64 to 0.54	0.58	−6 to −10	−7
4 with active malignancies	0.48 to 0.30	0.41	−16 to −20	−18

*For the meaning of Fract. A see text. TA means total albumin, GL globulin.

†For preparation of these lipids see text.

The data on cancer and noncancer sera are arranged separately in order of their Fract. A values in Tables IV and V. To facilitate the evaluation of the relations of the 3 protein fractions and to make a comparison possible with the previous clinical study,[1] Table VI was prepared by segregating the data of Tables IV *and* V *into 3 TA ranges (above 90 per cent, 70 to 89 per cent, and* 60 to 69 per cent of the average normal TA of 4.5 Gm. per 100 ml. serum) and computing the average Fract. A values of each range. The Fract. A values decrease in the lower TA ranges but the average ratios Fract. A/TA remain

TABLE V. FRACT. A, TOTAL ALBUMIN (TA) AND GLOBULIN (GL) IN SERA FROM 24 NONMALIGNANCIES

NAME	DIAGNOSIS	FRACT. A	TA	GL	FRACT. A $\frac{TA + GL}{TA}$
		(GM. IN 100 ML. SERUM)			
Ca	Latent syphilis	0.53	4.33	3.40	0.95
Ki	Neurasthenia	0.50	4.68	2.82	0.80
Ha	Normal	0.45	4.17	3.72	0.85
Ne	Thyroid disease	0.44	4.48	3.76	0.81
St	Uveitis	0.44	3.92	3.39	0.82
Me	Arthritis	0.43	4.49	3.52	0.77
Dod	Esophagitis	0.43	4.06	3.08	0.77
Ki	Adenoma of breast	0.41	3.16	3.80	0.90
Kr	Biliary cirrhosis	0.41	2.78	4.12	1.02
Do	Banti's syndrome	0.40	4.28	4.70	0.83
La	Uveitis	0.39	4.13	4.09	0.78
El	Thyroid disease	0.39	3.99	3.08	0.69
Ne	Thyroid disease	0.39	3.91	3.76	0.77
Do	Hypothyroid	0.38	4.13	2.59	0.62
Mo	Hypothyroid	0.36	3.59	3.70	0.73
Wa	Hypertrophy of prostate	0.35	4.26	4.13	0.69
Ba	Diabetes mellitus	0.34	3.81	3.56	0.66
Hu	Iritis	0.34	3.73	3.60	0.65
Ste	Cushing's syndrome	0.33	3.68	3.42	0.64
Car	Cirrhosis of liver	0.32	2.92	4.28	0.79
Schu	Stenosis of rectum; diverticulitis	0.25	3.12	4.40	0.61
She	Fibrosis of lungs	0.18	3.13	8.41	0.66
Fo	Abscess of gall bladder; diabetes, nephritis	0.10	1.88	4.36	0.33
	Average	0.37	3.78	3.88	0.75

TABLE V. FRACT. A, TOTAL ALBUMIN (TA), AND GLOBULIN (GL) IN SERA FROM 19 ACTIVE CARCINOMAS

NAME	TYPE	PRIMARY IN	FRACT. A	TA	GL	FRACT. A $\frac{TA + GL}{TA}$
			(GM. IN 100 ML. SERUM)			
Phi	Adeno.	Rectum	0.33	4.26	2.82	0.55
Du	Adeno.	Breast	0.32	4.39	2.56	0.51
Ga	Squamous cell	Soft palate	0.31	4.73	2.34	0.47
Tr	Adeno.	Breast	0.26	3.67	3.46	0.51
Bi	Adeno.	Breast	0.25	3.77	4.82	0.57
Eh	Adeno.	Breast	0.24	3.71	2.50	0.40
Le	Scirrhous	Stomach	0.24	2.67	4.00	0.60
An	Adeno.	Colon	0.22	2.94	1.76	0.35
B. J.	Adeno.	Sigmoid	0.21	3.82	4.46	0.45
Ro	Adeno.	Adrenal	0.21	3.15	2.82	0.40
Ma	Adeno.	Esophagus	0.21	2.64	3.84	0.52
Ab	Adeno.	Breast	0.19	2.76	4.00	0.47
La	Adeno.	Breast	0.18	2.75	2.00	0.31
Le	Adeno.	Breast	0.18	2.75	2.32	0.33
Je	Adeno.	Sigmoid	0.14	2.66	3.30	0.32
Fr	Adeno.	Breast	0.13	2.99	6.29	0.40
Pe	Adeno.	Lung	0.10	2.57	3.88	0.25
B. H.	Adeno.	Breast	0.10	2.52	3.20	0.23
Ho	Adeno.	Colon	0.10	1.89	2.52	0.23
	Average		0.22	3.15	3.34	0.45
Pi	Multiple myeloma		0.08	1.00	5.20	0.50
Ma	Adeno. Clinically no recurrence, one year after surgery	Breast	0.34	4.18	3.82	0.65

fairly constant in each group of sera. The products, Fract. A $\frac{TA+GL}{TA}$, show only small variations in the 3 TA ranges for each of the noncancer and cancer groups. After the effects of the globulin concentrations were corrected by calculating the products Fract. A $\frac{TA+GL}{TA}$ a clear separation of the noncancer and of the cancer sera becomes possible. As dividing point was chosen half of the sum of the grand averages of the products of the noncancer and of the cancer sera (Table VI) $\frac{0.77+0.44}{2}=0.605$. All 20 sera from active malignancies have lower products (Table V), and 23 of the 24 noncancer sera have higher products (Table IV). Allowing for ±5 per cent experimental error 4 sera (3 noncancer and 1 cancer) fall in the intermediate zone of 0.575 to 0.635.

TABLE VI. COMPARISON OF THE AVERAGE VALUES OF FRACT. A AND OF THE PRODUCTS FRACT. A $\frac{TA+GL}{TA}$ IN 3 TA RANGES FOR NONCANCER AND CANCER SERA

TA RANGE	FRACT. A* (GM IN 100 ML SERUM)	TA* (GM IN 100 ML SERUM)	GL* (GM IN 100 ML SERUM)	RATIO OF FRACT. A TO TA	FRACT. A $\frac{TA+GL}{TA}$ (GM IN 100 ML SERUM)
			Nonmalignancies		
Above 4.05	0.43	4.30	3.58	0.10	0.79
3.15 to 4.04	0.37	3.73	3.50	0.10	0.72
2.70 to 3.14	0.29	2.98	5.30	0.10	0.81
Average	0.36	3.67	4.13	0.10	0.77
			Malignancies		
Above 4.05	0.32	4.46	2.57	0.07	0.50
3.15 to 0.04	0.23	3.61	3.26	0.06	0.44
2.70 to 3.14	0.19	2.85	3.27	0.07	0.41
Average	0.25	3.64	3.03	0.07	0.44

*For the meaning of Fract. A see text. TA means total albumin. GL globulin.

TABLE VII. COMPARISON BETWEEN THE RESULTS OBTAINED ON SERA FROM NONMALIGNANCIES AND FROM MALIGNANCIES BY THE CENTRIFUGATION METHOD (CM) AND BY THE PAPER EXTRACTION METHOD (PM)

TA RANGE (GM IN 100 ML SERUM)	NONMALIGNANCIES			MALIGNANCIES		
	TA (GM IN 100 ML SERUM)	S CM (% OF NORMAL)	S PM (% OF NORMAL)	TA (GM IN 100 ML SERUM)	S CM (% OF NORMAL)	S PM (% OF NORMAL)
Above 4.05	4.30	88	88	4.46	65	69
3.15 to 0.04	3.73	75	78	3.61	47	57
2.70 to 3.14	2.98	59	58	2.85	39	40
Grand average	3.67	74	75	3.64	50	55

The averages for the Fract. A values in the 3 total albumin (TA) ranges in Table IV were transformed into S values, i.e. per cent of normal values by the formula $S = 100 \times \text{Fract. } A/0.49$. The S values for the same total albumin values were read from Fig. 1 of the previous paper.[1]

Comparison Between Evaluations of Results Obtained With the Centrifugation Method and With the Paper Extraction Method.—Since our experiments had to be interrupted we can report data only from 45 sera, analyzed with the centrifugation method. Our conclusions can be supported by the results on

578 sera analyzed previously with the paper extraction method.[1] Those data had been reported as S values, i.e., as per cent of the average Fract. S values of normal sera. To make comparable the results obtained with both methods, the Fract. A values from Table VI were calculated as S values, that is, per cent of normal by the formula 100 × Fract. A/0.49. Table VII shows that the average S values of the noncancer sera are very similar by both methods at each level of total albumin; while in the cancer sera the values by the centrifugation method are about 10 per cent lower than by the paper extraction method.

The Nature of Fract. A, a More Rapidly Extractable Albumin.—The magnitude of the Fract. A value of any serum gradually decreases as the time of standing of the mixture of serum with 38.2 per cent $(NH_4)_2SO_4$ is increased. After 1 hour the value is about 15 per cent greater, and after 48 hours about 10 per cent less, than the value at 19 hours. Even after 9 days, when the value has decreased about half, it continues to decrease. Also vigorous stirring or any elevation of temperature up to 35° C. decreases Fract. A values. It should be emphasized that such variations in time, agitation, or temperature have no significant effect on the total albumin value as measured by 31 per cent $(NH_4)_2SO_4$ or on the globulin values measured at 12 per cent $(NH_4)_2SO_4$, and this supports the idea that these decreases in Fract. A are not due to denaturation of protein.

The decreasing effect on the magnitude of Fract. A of increased duration of extraction and stirring demonstrates that Fract. A is not, as we formerly thought,[1, 5] a more soluble albumin, but rather a more rapidly extractable albumin fraction. The manner in which the presence of lipids or of globulin alters the *rate* of albumin solution is not clear, but the phenomenon undoubtedly involves interaction of albumin with lipids or albumin with globulin.

DISCUSSION

In the previous study a separation of the Fract. S values of cancer and noncancer sera was made in terms of a dividing line constructed from the average S values of each group at different TA levels.[1] In the present study the consideration of another factor, the globulin concentration, leads to a simpler and more accurate evaluation. One value, the product Fract. A $\frac{TA + GL}{TA}$, is used as dividing point for all sera. The data in Table II give an experimental basis for the use of this product. It represents the Fract. A values with correction for the effects of variations in the ratios GL/TA.

The centrifugation method described in this paper for estimation of Fract. A has the following advantages when compared with the paper extraction method which was used for measuring Fract. S. The use of paper is eliminated; so the effects of interaction between paper and proteins are avoided. The ratio of serum to extracting solution is increased and a smaller percentage of the albumin is extracted; these allow a more accurate measurement of the effects of some Fract. A altering substances. The effects of temperature variations on the amount of extracted protein are minimized. The data show less overlapping in the corrected values for the individual noncancer and cancer sera.

Disadvantages of the centrifugation method are the requirement of some special equipment and more time In some sera the supernatants A were turbid before heating, and the method could not be applied * We observed this phenomenon in 2 noncancer sera which were excluded from Table IV

The effects of the addition of various lipids to serum in vitro (Tables I, II and III) are best interpreted by assuming that the Fract S and Fract A values are influenced markedly by interactions between serum proteins and lipids One may conclude that in sera of the same albumin and globulin concentration, different Fract A values under various physiologic and pathologic conditions reflect variations in the concentration of Fract A-elevating or Fract A lowering lipids The lower than normal Fract A values usually observed in sera of diseased persons indicates either an increase in Fract A lowering or a decrease in Fract A elevating substances Further investigations are necessary to determine whether the still lower Fract A values in malignancies are caused by an increase in the concentration of the same kind of Fract A lowering substances commonly present in sera, or to the presence of specific lipids characteristic of malignant growth

We wish to emphasize that the described method is nonspecific and not a "cancer" test It is recommended not as a substitute but rather as a supplement to cytological methods Cytology reveals the presence of abnormal cells Serology measures chemical changes caused by their presence and is complicated by interfering factors related to other pathologic conditions We feel that analyses of a greater number of unselected sera from the same kinds of patients would not change significantly the statistical results To determine the clinical value of the proposed method for cancer diagnosis it seems more important to re examine with this method selected sera of patients with certain conditions in which "false positive" results had been obtained with previous methods, e g certain chronic or acute infections, liver and kidney diseases, and pregnancy The effects of food intake and of certain drugs (e g , sulfa drugs) have to be established The duration and magnitude of aftereffects of radiation and surgery on Fract A values must be determined before it will be possible to evaluate the significance of Fract A data on sera of treated cancer patients When information on these variables is available, the proposed method may be a useful aid in the diagnosis and prognosis of cancer

This method should be useful, along with other methods, in the characterization and isolation of Fract A lowering lipids or lipid protein complexes which are present during malignant growth If such lipids are found to be characteristic of certain or all kinds of malignancy, the significance would be great not only for serodiagnosis of cancer, but also for the metabolic nature of malignant growth

SUMMARY

1 A method is described in which the serum proteins are precipitated and then extracted successively with 38 2, 31, and 12 per cent ammonium sulfate solutions The extracts are separated from the precipitates by centrifugation at high speed, and their protein concentrations are measured turbidimetrically

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*See footnote on page 360

They are designated as Fract. A, B, and C, respectively. The sum of Fract. A *and Fract. B approximates the total albumin (TA) and Fract. C, the globulin* (GL).

2. With this method 45 sera (20 from malignancies) were analyzed. It was found that the products Fract. A $\frac{TA + GL}{TA}$ for all sera of patients with malignant disease were lower than a certain reference value, while those of patients without malignant disease, with one exception, were higher.

3. These results are compared with the evaluation of analyses on 578 sera in which the serum proteins had been precipitated onto strips of filter paper, and extracted with 38.5 and 31 per cent ammonium sulfate solutions. The advantages of the centrifugation method are discussed.

4. In vitro studies indicate that the characteristic differences between sera of patients with and without malignancies are due not to variations in the quantity or nature of the serum proteins, but to their interactions with certain nonprotein substances. Mixing sera with certain fatty acids causes elevation of their Fract. A values. Lower Fract. A values are observed after mixing sera with most bile acids, with certain steroids and other substances related to sex hormones, or with certain acetone-soluble lipids extracted from cancer sera.

5. These observations suggest that during malignant growth certain characteristic lipids or lipid-protein combinations occur in the serum.

6. The clinical value and the limitations of the described method for serologic cancer diagnosis and prognosis are discussed.

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CHROMOGENS IN THE URINE OF NORMAL INDIVIDUALS AND OF EPILEPTIC PATIENTS WHICH REACT WITH EHRLICH'S ALDEHYDE REAGENT

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THE test for porphobilinogen described by Watson and Schwartz[1] in 1941 has gained widespread acceptance as a simple laboratory aid in the diagnosis of acute intermittent porphyria. Hammond and Welcher[2] concluded that the test was quite specific.

For several years I was interested in following a number of patients with acute intermittent porphyria.[3] As a result of this interest, 2 patients were referred from the Neurology-Epilepsy Clinic. These patients had been treated for epilepsy for over 5 years, and had recently developed acute, intermittent abdominal pain. The diagnosis of acute intermittent porphyria was entertained, especially when a test of their urine for porphobilinogen revealed a purplish-pink color which was insoluble in chloroform. However, no uroporphyrin and no increase in coproporphyrin could be detected in several 24-hour specimens of the patients' urine.

Two other possibilities came to mind. First, that the barbiturates used in the treatment of epilepsy might have caused some aberration in pyrrole metabolism This would fit nicely with the clinical observation of Waldenstrom[4] that barbiturates may cause exacerbations of acute intermittent porphyria, and the experimental evidence described by Goldberg[5] on the effect of certain barbiturates on the porphyrin metabolism of rabbits. The second possibility derived from the observation that a considerable number of patients with acute intermittent porphyria have epileptiform seizures. If the urine of patients with epilepsy contained porphobilinogen, then perhaps there was also a defect in pyrrole metabolism in epilepsy. (In this connection, Goldberg and his associates[6] have recently discussed the lack of physiologic activity of purified porphobilinogen.)

This is a report concerning the frequent finding of a positive test for porphobilinogen in the urine of epileptic patients, and attempts to characterize the chromogen responsible.

METHODS

The test for porphobilinogen was performed as described below. Five milliliters of Ehrlich's aldehyde reagent (as modified by Watson) was added to 5 ml. of fresh urine. Ten milliliters of a saturated solution of sodium acetate was then added. Finally, 5 ml. of

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chloroform was added. The mixture was shaken well after each addition. The test was considered negative if no pink color remained in the aqueous phase. Only a definite purplish-pink color was considered positive. Reagents from several different sources were used with identical results.

Later in the study, the Vahlquist type of Ehrlich's aldehyde reagent was used. This necessitated filtering after the addition of the saturated solution of sodium acetate.

The method used to concentrate the Ehrlich aldehyde reacting substances in the urine was a modification of the methods of Westall,[7] and of Cookson and Rimington[8] for the crystallization of porphobilinogen.

Urine specimens were collected daily for 3 to 6 days from 5 normal individuals (fellow laboratory workers, all men, and under age 35) and 5 epileptic patients.* Each day's specimen was acidified with acetic acid, and then mercuric acetate (15 per cent w/v) was added until precipitation was complete. The precipitate was collected and washed with mercuric acetate (1 per cent w/v). The daily specimens were combined, resuspended in distilled water and decomposed by H_2S. After filtration and aeration, excess lead acetate (10 per cent w/v) was added. The deeply pigmented precipitate was removed and silver nitrate (20 per cent w/v) was added to the filtrate until precipitation was complete. The silver nitrate precipitate was removed and mercuric acetate (15 per cent w/v) was again added to the filtrate. This mercury precipitate, almost white, was collected, resuspended in a small amount of distilled water, and decomposed with H_2S. After filtration and aeration, the filtrate was passed through an alumina column, approximately 3 cm. in diameter, and 20 cm. in length, prepared by washing with 3 per cent acetic acid followed by 1,500 ml. of water. After all the filtrate had been put on the column, water was then passed through until no material giving a color with Ehrlich's aldehyde reagent appeared in the eluate. Ammonia, 0.1 N, was then run through the column, at a rate not exceeding 100 ml. per hour. The Ehrlich aldehyde reacting material in the eluate was collected on a fraction collector. The fractions were then lyophyllized, taken up in 5 ml. of 0.1 N ammonia, and then frozen.

Paper chromatograms were made by the ascending method and were run 4½ to 6 hours at room temperature. The solvent was a mixture of butanol, acetic acid, and water (using the top layer only) as described by Partridge.[9] The paper chromatograms were dried at room temperature and then sprayed with Vahlquist type Ehrlich's aldehyde reagent.

Urine specimens from two patients with porphyria hepatica, acute intermittent type, were prepared in the same manner and used for comparison of porphobilinogen with the chromogens present in the samples from epileptic patients.

RESULTS

Table I gives the results of the Watson-Schwartz tests in 91 patients from the neurology clinic.

Fourteen of these 91 epileptic patients (those with the most strongly "positive" tests for porphobilinogen) were then examined as to their liver function by means of Bromsulphalein excretion, thymol turbidity, cephalin flocculation, and serum bilirubin. All values for these tests were within normal limits except for a mild reduction in Bromsulphalein excretion in one patient. Twelve of the fourteen still had "positive" tests for porphobilinogen.

In the patients tested, there was no correlation of "positive" tests with the type of epilepsy, drug used, or age and sex of the patient. The only possible exception was a group of 5 patients with petit mal epilepsy alone, none of whom had a "positive" test. The drugs used in the treatment of these patients included phenobarbital, Dilantin, Tridione, Mesantoin, Mebaral, Milontin, Diamox, Phenurone, Mysoline, Eliptan, and Hibicon.

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Spectrophotometric studies of the purplish pink color obtained when the urine was tested for porphobilinogen showed an abrupt increase in light transmission at 580 mμ, and a long, relatively flat plateau from 500 to 580 mμ, quite distinct from the characteristics of porphobilinogen aldehyde (552 and 522 mμ)

TABLE I PATIENTS WITH EPILEPSY*

CLASSIFICATION	NUMBER OF PATIENTS	NUMBER OF TESTS "POSITIVE"
Total Patients	91	47
Male	54	33
Female	42	15
Under age 14	18	7
Over age 50	4	2
Petit mal	5	0
Petit mal + grand mal	26	11
Grand mal + psychomotor	39	25
Psychomotor	10	5
All other epilepsy	11	6

*Diagnoses are the result of a correlation of clinical and electroencephalographic findings

The urine of the 5 epileptic patients at Dixon State Hospital and of the 5 fellow laboratory people was then treated by the method described above In this attempt to concentrate and then characterize the chromogen responsible for the "positive" tests for porphobilinogen in the epileptic patients, three *major fractions were obtained The first, the water fraction, contained chromogen* which would not adhere to the alumina column and was collected by washing the alumina column with water The second fraction appeared at a pH of 4 to 6 when the column was washed with dilute ammonia The third fraction was easily seen traveling down the alumina column as a brownish ring and appeared in the eluate at a pH of 7

The water fraction gave a pinkish purple color upon the addition of Ehrlich's aldehyde reagent The addition of saturated sodium acetate solution made the color more purple This purple color was insoluble and light absorption studies still showed a plateau from 540 to 570 mμ Upon standing for several days at room temperature a grayish color developed

The pH 4 to 6 fraction was found in 4 out of 5 normals but not in the epileptic patients It produced an orange brown color with Ehrlich's aldehyde reagent and became yellowish upon the addition of saturated sodium acetate solution Spectrophotometric study of this pigment showed a plateau from 470 to 500 mμ

The pH 7 fraction gave a purplish color upon addition of Ehrlich's aldehyde reagent, and addition of a saturated solution of sodium acetate produced a faint light blue color

Ascending paper chromatograms were then made, using all of the fractions As a control, the pH 7 fraction from a patient with acute intermittent porphyria was used This fraction contained porphobilinogen and the aldehyde color gave a peak at 552 mμ The purplish pink spot of porphobilinogen aldehyde appeared immediately upon spraying with aldehyde reagent When very small spots were made, this material had an R F of 0 51 The water fraction, in both normal and epileptic patients, yielded a purple spot with an R F of 0 50 This purple spot appeared slowly after the paper was sprayed with the aldehyde reagent, and did not reach maximum intensity until about 15 minutes. A few hours later the spot became yellow and 24 hours later it was a blue-green.

The pH 4 to 6 fraction, found in 4 out of 5 normal urines but not in the urine of epileptic patients, yielded two spots, both orange-brown. The larger spot had an R.F. of 0.46 and appeared within one minute after spraying with the aldehyde reagent. Under ultraviolet light, this spot fluoresced a bright pinkish-red reminiscent of uroporphyrin. The smaller spot appeared slowly and had an R.F. of 0.34 and did not fluoresce.

The pH 7 fraction contained three spots in 3 out of 5 normal urines, but only two spots in all the urines of epileptic patients, as well as in two of the normal urines. All of these spots appeared slowly after spraying with the aldehyde reagent and reached maximum intensity over an hour later. Two of the spots were purplish and faint and had R.F. values of 0.95 and 0.78. The third spot, present in 3 out of 5 normals only, was most prominent. It was deep blue and had an R.F. of 0.86.

In two patients with acute intermittent porphyria, the same fractions present in the urine of normal patients were found, in addition to the porphobilinogen in the pH 7 fraction. In two additional patients with epilepsy, who were having frequent seizures when their urine was collected, very little material giving any color with Ehrlich's aldehyde reagent could be found. In one of these, no such pigment could be demonstrated at all, and in the other, only a small water fraction, but no pH 4-6 or pH 7 fractions could be found.

Under ultraviolet light a great many small spots and bands appeared in all of the fractions. Before spraying with aldehyde reagent many of these had a light bluish fluorescence. After spraying with aldehyde reagent only the porphobilinogen spot from the urine of patients with acute intermittent porphyria and the spot in the pH 4-6 fraction with an R.F. of 0.46 gave a pink fluorescence which looked like uroporphyrin.

DISCUSSION

The Watson-Schwartz test of the urine of epileptic patients was suggestive of a positive test in roughly 50 per cent of patients tested. Results of concentration of urine and chromatography of the concentrated fractions failed to reveal any porphobilinogen of the type found in acute intermittent porphyria. However, several chromogens were present in the concentrated urine of both normal and epileptic patients which could be confused with porphobilinogen upon the addition of Ehrlich's aldehyde reagent. Possibly, a quantitative difference exists in the chromogens present in the urine of normal versus epileptic patients which would account for the mildly positive-appearing tests in the unconcentrated urine of epileptic patients. It also seems likely that the amount of chromogens excreted in the urine of epileptic patients varies widely from time to time. The only qualitative differences were the absence of the pH 4-6 fraction from the urine of epileptic patients and also the absence of the R F 0 86 portion of the pH 7 fraction of the urine of these same patients These fractions were found in the urine of normal patients

The chromogens responsible for the colors when aldehyde reagent was added have not been identified They are distinctly different from porphobilinogen, and may be indoles or tryptophane[10] These chromogens may be a reflection of a metabolic defect causing disturbances in central nervous system function

SUMMARY

1 The test for porphobilinogen described by Watson and Schwartz was done on the urine of 91 epileptic patients

2 Forty seven of these 91 patients had a definite "positive" test

3 Studies of the chromogens, causing these positive appearing tests failed to identify them, but did show them to be distinct from porphobilinogen aldehyde

4 The significance of these chromogens is unknown The suggestion is made that these chromogens may be related to a metabolic defect in epileptic patients

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ENDOCRINE FACTORS IN THE ALTERATIONS OF THE BLOOD COAGULATION MECHANISM FOLLOWING SURGERY

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EVIDENCE that the transient thrombocytopenia and depressed thrombocytogenesis characteristic of the early postoperative period[1] can be simulated by ACTH-induced "dummy stress" in normal individuals[2] suggests that adrenocortical hyperactivity may be a major factor in the altered mechanisms of blood coagulation which have been observed following surgery.[3] The potential importance of these alterations in the pathogenesis of thromboembolism prompted the present correlative evaluation of adrenocortical function and the clotting mechanism in surgical patients.

MATERIALS AND METHODS

The subjects for this study were patients on the general surgical service, undergoing elective surgical procedures of varying degrees of magnitude. None had known hematologic abnormalities. All convalesced uneventfully, without thromboembolic sequelae.

Serial observations were made pre- and postoperatively of peripheral blood platelet and eosinophil counts, coagulation times, plasma and serum prothrombin times, serum "free" 17-OH corticoid concentrations, and bone marrow megakaryocyte activities.

Blood samples were obtained by venipuncture, bone marrow by aspiration from vertebral spinous processes in the upper lumbar or lower thoracic region.

Platelets were counted by the direct method, using modified Rees-Ecker diluting fluid.[4] Absolute eosinophil counts were made by the technique of Henneman and associates.[5]

Plasma prothrombin times were determined by the method of Quick. The activity of prothrombin accessory factors was estimated by comparison of the prothrombin times of aliquots of plasma with and without the addition of material providing an excess of these factors.[6]

Residual prothrombic activity of serum was determined by the technique previously described[7]; the results are expressed in terms of the clotting time (in seconds) of the reconstituted system.

Concentrations of unconjugated 17-OH corticoids in serum were measured by the method of Nelson and Samuels.[8]

Megakaryocyte productivity was assessed by examination of stained marrow smears, utilizing a system of differential classification[1] in which megakaryocytes are grouped according to platelet content. With the exception of units whose cytoplasm is made up entirely of platelets, the percentage of platelet-containing cells is assumed to indicate productivity.

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RESULTS

As shown graphically in Figs 1 and 2, the subject material has been considered in two groups, 10 patients subjected to relatively minor surgical procedures (such as inguinal herniorrhaphy), and 12 patients undergoing major operations (subtotal gastrectomy, pulmonary or cardiac surgery)

Group I (Major Surgery) —

Platelets. Reduction in peripheral blood platelet count during the operative procedures averaged 30 per cent Return to preoperative range was evident on fourth postoperative day, and the ensuing thrombocytosis exceeded the preoperative mean by 45 per cent

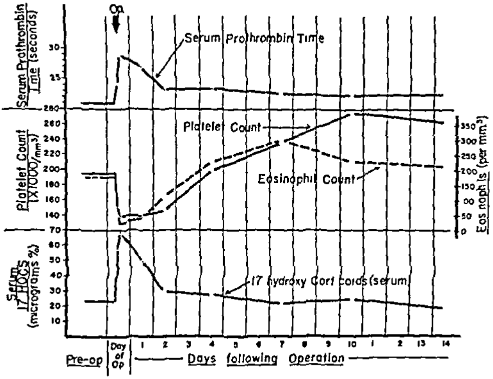

Fig 1 —The effects of major surgery on serum corticoids eosinophil and platelet counts and serum prothrombin time Mean data from 12 patients are represented

Eosinophils Fluctuations were similar to platelet response, except that eosinopenia was more transient and "rebound" eosinophilia occurred earlier (seventh day) in the postoperative period

Plasma prothrombin times One stage prothrombin times were prolonged for ten days following operation This trend was maximal (64 per cent of normal) on the first postoperative day and apparently represented a reduction in the activity of prothrombin accessory factors

Megakaryocyte productivity The ratio of platelet producing to nonproducing cells fell from 1.15 $(=\frac{44.6}{38.8})$ before operation to 0.33 $(=\frac{10.1}{30.3})$ at the end of operation, rising to 2.0 $(=\frac{39.0}{19.7})$ on the tenth postoperative day

Serum prothrombin times Prompt elevation was noted, maximal at the end of operation, with normal values on and after the second postoperative day

Serum 17 OH corticoids Rise to highest level was attained at the end of surgery, with values in normal range on and after second day

Group II (Minor Surgery).—The patterns of change following minor operations (Fig. 2) differ in degree rather than in kind from the responses to more major procedures.

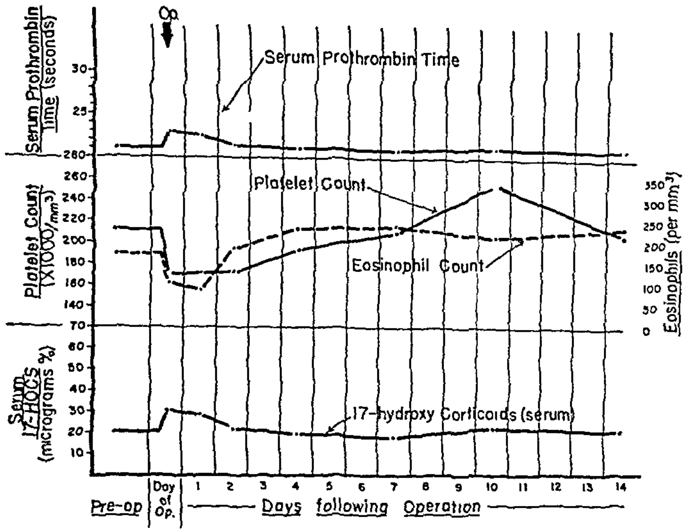

Fig. 2.—The effects of minor surgery on serum corticoids, eosinophil and platelet counts, and serum prothrombin time. Mean data from 10 patients are represented.

DISCUSSION

Acute changes occur in the clotting mechanism as well as in the peripheral level of 17-OH corticoids following surgery. The attainment of maximal corticoid levels correlates well in time with the attainment of maximal alterations in the elements of the clotting mechanism included in this report. However, with the exception of the serum prothrombin times, beyond this initial phase of the responses no direct correlation is evident between corticoids and clotting factors.

The operative rise in corticoid level was invariably accompanied by a fall in platelet count and in megakaryocyte productivity. However, in individual patients no systematic relationship was evident between the magnitudes of the corticoid response and the degrees of thrombocytopenia and depressed thrombocytogenesis. Observations not included in this report indicate, furthermore, that thrombocytopenia (and depressed platelet production) without elevation in circulating corticoids occurs occasionally after simple intravenous infusions of dextrose solutions in normal individuals. From these observations it appears unlikely that the corticoid response to surgical stress is an important factor in initiating alterations in platelet level and platelet production.

Thrombocytosis and increased megakaryocytic productivity in the second week of convalescence, found in approximately two-thirds of these patients, was not accompanied by changes in peripheral blood corticoid levels.

The correlation between the fluctuations in corticoid concentration and the efficiency of prothrombin consumption in individual patients was consistent both in timing and in magnitude. This correlation suggests that adrenocorticoids may be the determining influence in this "superconsumption" of prothrombin, suggestive of increased activity in the thromboplastin mechanism, in the early phase of the response to stress

SUMMARY

1 Observations were made of peripheral blood platelet and eosinophil counts, megakaryocyte productivity, plasma and serum prothrombin times, and serum 17 hydroxycorticoid concentration in patients undergoing elective surgical operations of varying degrees of magnitude

2 Early postoperative decreases were observed in platelet and eosinophil levels, in platelet production, and in the activity of prothrombin accessory factors, these changes correlated well temporally with the observed increase in peripheral corticosteroid levels, but in each instance the alterations in these elements of the clotting mechanism persisted for several days after return of the steroid level to preoperative range

3 Late postoperative thrombocytosis and augmented platelet production were not associated with measurable alterations in adrenocortical activity

4 Good correlation was observed between prothrombin consumption and corticoid level throughout the periods of study

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PLASMA AND RED CELL CHOLINESTERASE IN 800 "HEALTHY" BLOOD DONORS

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THE past few years have seen a rapid increase in the number and types of anticholinesterase agents used as medications and as commercial insecticides. Great interest has also been shown in the development of anticholinesterase agents as poison gases and of effective antidotes to their use. In addition, cholinesterase activity has been utilized as an index of disease and prognosis in hepatitis and other diseases of the liver[1-11] and in ulcerative colitis.[9, 12]

Because of the growing interest in cholinesterase, it has become increasingly important to establish "normal values" for plasma and red cell cholinesterase in man. "Normal" cholinesterase levels have been reported by a number of investigators, such as Callaway and associates,[13] Sealey,[14] Fremont-Smith and associates,[10] Huerga and associates,[15] Wolfsie and associates,[16] Reinhold and associates,[17] Vorhaus and associates,[18] Kaufman,[19] Molander and associates,[20] Oka,[21] and Sabine.[22] The majority of these studies, however, were based on cholinesterase determinations by various methods in rather small groups of subjects, consisting primarily of men, in a narrow age range.

The unusual opportunity* to use blood specimens from blood-bank donors made it possible to undertake cholinesterase determinations in a large series of normal subjects with a wide age range. The purpose of this study was to establish normal values for plasma and red cell cholinesterase with respect to age and sex, to establish any variations in these values between individuals and in the same individual at different times, and to determine whether a relationship exists between plasma and red cell cholinesterase.

MATERIALS AND METHODS

Subjects.—The specimens used in this study were obtained over a 5-month period from 400 male and 400 female blood donors. The criteria of acceptance as blood donors had eliminated persons with any of the following: a history of jaundice, syphilis, tularemia, undulant or relapsing fever, diabetes, peptic ulcer, or gonorrhea; a transfusion of blood or

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its derivatives within the past year, household contact with jaundice within one year or poliomyelitis within 3 weeks, infectious mononucleosis during the preceding 2 years, a history of rheumatic fever or tuberculosis, unless mild and of short duration, pregnancy, unless terminated 6 months previously, colds sore throats, or infections with fever (above 99 2° F) Persons with a temperature of 99 6° F without other symptoms were accepted Subjects with a history of asthma or allergy could be accepted at the discretion of the physician A maximum pulse rate of 110 per minute and blood pressures ranging from 110/60 to 200/100 were acceptable Serologic tests for syphilis were negative in all subjects Hemoglobin levels in all cases were 12 Gm or above (as determined by the copper sulfate screening method) In addition to the above criteria, no known or suspected alcoholic and no person whose appearance suggested possible malnutrition or recent disease was accepted Less than 10 per cent of the entire group were 'professional blood donors These are screened much more carefully than the ordinary donors, especially with regard to general health and history of present or past disease

The donors ranged from 18 to 60 years of age They were separated into groups according to age by year To insure wide age spread, the groups for each year were limited to not more than 12 individuals of the same sex Some selection inevitably occurred since the groups in the median years and the quota of men were completed first It was more difficult to complete the groups at the extremes of the age range and to fill the quota of women

Method—Blood was drawn from fasting donors into 15 ml graduated centrifuge tubes containing a small quantity of dry heparin * The tubes were centrifuged, the plasma was removed by aspiration, and the red cells were washed twice with 0 9 per cent sodium chloride solution The plasma and R B C cholinesterase determinations were made within 24 hours, using Michel's potentiometric method[2,3]† which measures the fall in pH occurring when acetylcholine chloride‡ is split by cholinesterase into acetic acid and choline The cholinesterase activity is recorded as the change in pH produced by 0 02 ml of buffered R B C or plasma with the acetylcholine substrate in one hour at 25° C, Δ pH per hour Corrections are made for nonenzymatic hydrolysis and variations in the rate of pH change with a decrease in pH

RESULTS

1 *Accuracy of Method*—An experiment was conducted to assess the accuracy of our cholinesterase measurements and the constancy of the cholinesterase level in an individual over short periods of time A blood sample was taken once a week for 3 weeks from each of 10 healthy subjects Each sample was divided into 3 parts, which were analyzed separately It was possible to estimate (1) the standard error of each analysis by comparing the triplicate analyses of the same blood sample, and (2) the standard error for the temporal variation of the cholinesterase level in the individual from week to week by comparing the mean values for the 3 blood samples and allowing for the variability of these means

The results were as follows *standard error of analysis,* R B C cholinesterase, 0 022 ± 0 002, plasma cholinesterase, 0 022 ± 0 002, *standard error of temporal variation,* R B C cholinesterase, 0 011 ± 0 004, plasma cholinesterase, 0 033 ± 0 006 These standard errors are negligible when compared with the interindividual variations

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*Supplied by The Upjohn Company Kalamazoo Mich

†Although several methods are available to determine plasma and R B C cholinesterase we selected Michel's potentiometric method because it is simple quick highly accurate and gives reproducible results

‡Supplied by Merck & Co Inc Rahway New Jersey

2. The Relationship Between Plasma and R.B.C. Cholinesterase.—Although these two esterases differ, in that each has its own specific substrate, they do have in common the ability to hydrolyze acetylcholine. It is not known whether the two esterases are physiologically interdependent. A correlation has been suggested, however, in a study of ulcerative colitis,[12] where it was found that the serum cholinesterase level was low and the R.B.C. cholinesterase level high when the disease was active; whereas with healing the serum cholinesterase rose and the R.B.C. cholinesterase fell.

Analysis of the present data, however, showed little relationship between the plasma and R.B.C. cholinesterase levels in the same individual; the correlations were 0.087 ± 0.050 for men and 0.016 ± 0.050 for women. This suggests that the physiologic mechanisms that determine the two cholinesterase levels are not closely related.

TABLE I. CHANGE IN PLASMA CHOLINESTERASE LEVEL WITH AGE

MEN				WOMEN			
AGE	UPPER LIMIT	LOWER LIMIT	MEAN	AGE	UPPER LIMIT	LOWER LIMIT	MEAN
20	1.35	0.48	0.91	20	1.17	0.30	0.74
30	1.37	0.50	0.93	30	1.21	0.34	0.78
40	1.39	0.52	0.95	40	1.25	0.38	0.82
50	1.41	0.54	0.97	50	1.29	0.42	0.86
60	1.43	0.56	0.99	60	1.33	0.46	0.90

3. The Relationship of Plasma and R.B.C. Cholinesterase to the Age of the Donor.—It was found that the R.B.C. cholinesterase level did not change with age in either men or women. The plasma cholinesterase level, however, showed a definite, although small, increase with age in both sexes, amounting to 0.002 ± 0.001 Δ pH per hour per year of age in men and 0.004 ± 0.001 per year in women (Table I). This does not mean that the plasma cholinesterase levels of all individuals would necessarily increase at these rates as they grew older. For example, perhaps plasma cholinesterase levels are slightly higher in healthy than in less healthy persons, and perhaps older blood donors, as a class, are in exceptionally good health. These suppositions would explain our findings without indicating that the plasma level is actually increased with age.

4. The Relationship of Plasma Cholinesterase and R.B.C. Cholinesterase to Sex.—The levels of both cholinesterases were higher in the men than in the women donors. The difference of means was definitely significant for the plasma level (0.136 ± 0.020), but not significant for the R.B.C. level (0.016 ± 0.011).

5. The Relationship of Plasma and R.B.C. Cholinesterase to Day or Month "Batch Effects."—The samples were collected and analyzed in batches of not more than 40 a day over a period of 5 months. The variations in results from batch to batch were negligible compared to the individual variations within batches. At least 97 per cent of the total variation remains if batch differences are entirely eliminated. Since the comparisons on the basis of age and sex were made within batches, they are not dependent on any assumption of constancy from batch to batch.

TABLE II. ESTIMATED MEANS, STANDARD DEVIATIONS, AND NORMAL RANGE OF CHOLINESTERASE LEVELS FOR AGE 40

	MEN	WOMEN
*R.B.C. Cholinesterase**		
Mean	0.766 ± 0.007	0.750 ± 0.008
Standard deviation	0.081 ± 0.008	0.082 ± 0.008
Range		
Upper limit	0.95	0.94
Lower limit	0.58	0.56
*Plasma Cholinesterase**		
Mean	0.953 ± 0.012	0.817 ± 0.017
Standard deviation	0.187 ± 0.019	0.187 ± 0.019
Range		
Upper limit	1.39	1.25
Lower limit	0.52	0.38

*Values expressed in Δ pH per hour per 0.02 ml. red cells or plasma.

6. *Estimates.*—The estimated means and standard deviations for plasma and R.B.C. cholinesterase in male and female donors aged 40 years are given in Table II.

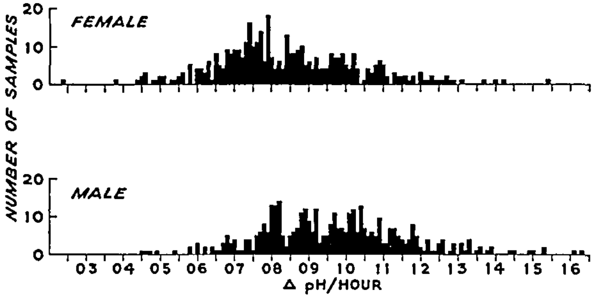

Fig. 1.—Curve showing distribution of plasma cholinesterase in 800 healthy blood donors (400 women and 400 men).

7. *Limits of Normality.*—Assuming that our blood donors are a sample of normal healthy persons and that cholinesterase levels follow the normal distribution, we can establish limits of normality of cholinesterase levels. Table II shows the normal range of R.B.C. and plasma cholinesterase levels according to sex in the donors aged 40, as calculated from our data. Ninety-eight per cent of the levels found in each group are estimated to fall between the limits shown, with 1 per cent below the lower limit and 1 per cent above the upper limit. Since the plasma but not the R.B.C. level varies slightly with age, the values for plasma cholinesterase in men should be increased about 0.02 per decade above 40 and decreased about 0.02 per decade below 40, in

women the rate of increase is 0.04 per decade. Figs. 1 and 2 show the distribution of plasma cholinesterase and of red blood cell cholinesterase in the 800 "healthy" blood donors in the present series.

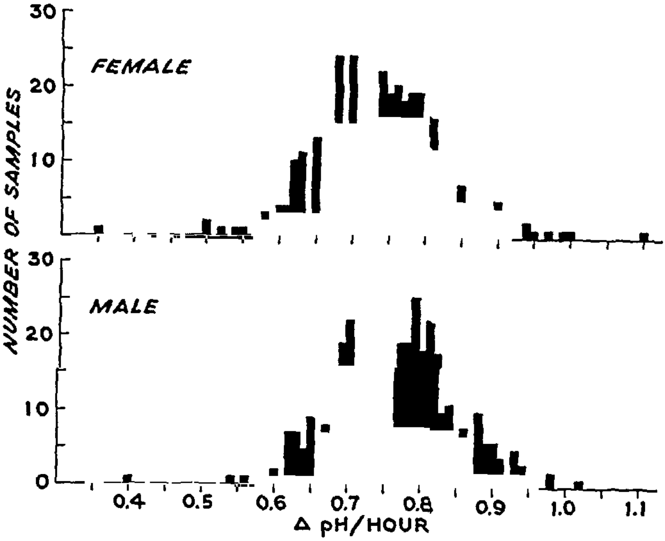

Fig. 2.—Curve showing distribution of red cell cholinesterase in 800 "healthy" blood donors (400 women and 400 men).

DISCUSSION

The earlier investigations are difficult to evaluate because of differences in methods for determining cholinesterase levels, in criteria of normalcy, and in selection of subjects. In many cases the groups tested were comprised largely of men, and little effort was made to obtain a wide age spread. In some of these studies cholinesterase levels were determined on serum rather than plasma; however, since the esterase activities of the two are identical, the data are comparable. Despite these differences, comparison of our results with those previously reported seems pertinent.

1. Results of Other Authors.—Vorhaus and Kark[18] determined the serum cholinesterase in 120 healthy individuals and found that the range of activity was 0.58 Δ pH per hour to 1.37 Δ pH per hour, with a mean of 0.94 and a standard deviation of 0.16. They concluded that differences in enzyme activity did not correlate with age, weight, sex, height, or surface area and that, although the cholinesterase activity varied widely from one healthy individual to the next, the level was "remarkably constant for each healthy, well-fed individual from day to day and from month to month."

Fremont-Smith, Volwiler, and Wood[10] found a mean serum cholinesterase activity of 0.95 Δ pH per hour, with a standard deviation of 0.24, in 20 normal men, whereas in 20 normal women the mean was 0.78, with a standard deviation of 0.12.

Wolfsie and Winter,[16] using a microadaptation of Michel's potentiometric method, tested 255 healthy male subjects and found the range for plasma cholinesterase was 0 408 to 1 652 Δ pH per hour, with a mean of 0 912 and a standard deviation of 0 112 The range for R B C cholinesterase was 0 554 to 1 252, with a mean of 0 861 and a standard deviation of 0 091 Δ pH per hour

Sealey,[14] in a study of 67 men and women, reported a mean plasma activity of 0 92 Δ pH per hour, with a standard deviation of 0 20, and a mean R B C activity of 0 773, with a standard deviation of 0 09 He stated, however, that 40 of these subjects had been minimally exposed to organic phosphate compounds

Callaway, Davies, and Rutland,[13] using the Warburg technique, studied a group of 247 healthy adults, 66 of whom were women They concluded that "sex, age, occupation, and season are without effect upon the level of plasma enzyme But, in the case of the red cell enzyme, a group of servicemen were found to have a significantly higher mean level than a male civilian population of comparable age It is conceivable that this may be due to the active outdoor life led by all of the first group, together with a high general level of physical fitness "

Reinhold, Tourigny and Yonan,[1] using a photometric indicator method, found that the serum cholinesterase activity was higher in normal men than in women Using a titration method, Kaufman[19] made the same observation

2 Comparison of Our Results With Those of Other Authors—The results of Fremont Smith, Volwiler, and Wood[10] are in close agreement with our findings, although they did not measure R B C cholinesterase

The conclusion of Callaway et al [13] that age has no significant effect on the R B C cholinesterase level in either sex agrees with our findings Their statement that sex and age have no effect on the level of plasma enzyme is contrary to our results The latter conclusion may be explained by the fact that they tested a smaller number of subjects and apparently examined age dependence by graphic analysis alone, which could not be expected to detect the small increase with age found in our group Their results also showed a somewhat higher mean cholinesterase level in men (1 87 per cent for R B C and 2 6 per cent for plasma) than in women

Our conclusion that plasma cholinesterase levels are correlated with sex and to a small extent with age are at variance with those of Vorhaus and Kark [18] A detailed comparison cannot be made since these authors did not give the age range and sex distribution in their group Their mean of 0 94 for serum cholinesterase is similar to our mean of 0 95 for men aged 40

Our plasma cholinesterase values are somewhat higher and R B C values somewhat lower than those reported by Wolfsie and Winter [16] Again, since they did not give the age range of their subjects, we cannot make an accurate comparison of plasma values in the two studies The higher R B C levels found by these authors might be explained by the possibility that, since they did not wash the red cells, some plasma may have contaminated the assay

SUMMARY

R.B.C. and plasma cholinesterase levels were determined in 800 normal, "healthy" blood-bank donors (400 men and 400 women), ranging from 18 to 60 years of age. The subjects were divided into groups according to age by year. Each group was limited to 12 individuals or less of the same sex. Blood samples were analyzed by the Michel potentiometric method in batches of not more than 40 a day over a 5-month period. The values from batch to batch were relatively constant.

Analysis of the data showed: (1) no relationship between R.B.C. and plasma cholinesterase levels; (2) a small increase in plasma cholinesterase with age; (3) no effect of age on the R.B.C. cholinesterase level; and (4) the levels of both plasma and R.B.C. cholinesterase were higher in men than in women; the difference in R.B.C. levels was not significant.

Normal cholinesterase values established by this method were as follows:

		Range (Δ pH per hour)	Mean (Δ pH per hour)
R.B.C. cholinesterase			
	Men	0.58-0.95	0.766
	Women	0.56-0.94	0.750
Plasma cholinesterase (for age 40)			
	Men	0.52-1.39	0.953
	Women	0.38-1.25	0.817

The plasma values increase slightly with age.

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THE EFFECTS OF CORTISONE ON THE GALACTOSE TOLERANCE TESTS OF NORMAL MEN

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PREVIOUSLY we reported the effects of a single oral dose of 200 mg. of cortisone acetate upon intravenous fructose and glucose tolerance tests in normal men.[1] It was observed that cortisone resulted in impaired glucose tolerance but did not affect the fructose tolerance test in the same subjects.

The present study was designed primarily to determine if galactose tolerance is altered by a single large dose of cortisone. In addition, it was hoped that more information might be obtained concerning the site of cortisone action in intermediary metabolism in view of the fact that galactose enters the Embden-Meyerhof scheme at a level different from either fructose or glucose (Fig. 1).

METHODS

An intravenous galactose tolerance test was performed on two separate occasions in each of the 11 normal men receiving a regular hospital diet containing at least 250 Gm. of carbohydrate and 3,000 calories daily. In the first instance, the galactose was administered without antecedent cortisone; a few days later, the galactose was given 4 hours after the oral ingestion of 200 mg. of cortisone acetate. Immediately after an initial sample of venous blood had been obtained, the galactose was given intravenously over a 4-minute interval as a 50 per cent solution in an amount calculated to provide 0.5 Gm. galactose per kilogram of body weight. Venous blood was obtained every 15 minutes for 90 minutes after the injection and a final blood sample was drawn at 120 minutes. The experiments were begun at 8 A.M. with the subject fasting and recumbent throughout the study. Approximately 10 hours before each test each subject received 50 Gm. of glucose orally as a supplement to his regular diet.

Blood galactose was determined as follows. A 2 ml. sample of whole oxalated blood was shaken for 30 minutes at room temperature with 10 ml. of 12 per cent washed baker's yeast. Deproteinization was accomplished by the addition of zinc sulfate and barium hydroxide. Two milliliters of copper reagent were added to aliquots (0.5 to 2.0 ml.) of the blood filtrate and the tubes placed in a bath of boiling water for twenty minutes.[2] The blood obtained prior to galactose administration was similarly treated to provide a "blank" value. The determination was completed by the Nelson procedure.[3] Total blood hexose was determined by the same procedure, allowing twenty minutes for the copper reduction with omission of the yeast fermentation.[2] Galactose and glucose standards were similarly treated. The calculation of blood glucose concentration was taken as the difference between total hexose and galactose concentrations, making appropriate allowance for the different reducing powers of the two sugars as determined by their simultaneous measurement using standard solutions.

Urine was collected from the beginning of the infusion period to the end of the study and its sugar content determined by the semiquantitative method of Somogyi.[4]

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RESULTS

1. The galactose tolerance tests in the eleven subjects, with and without antecedent cortisone administration, are listed in Table I and the mean curves depicted in Fig. 2. With the exception of the 45-minute blood sample, there is no statistically significant difference in the blood galactose levels of the control experiments and those following cortisone administration (i e., according to the "t" test using paired comparisons P is greater than 0.1). At 45 minutes after the end of the injection, the difference between mean blood galactose levels with and without antecedent cortisone administration has a P value of < 0.01 using the same statistical methods. However, when the rate of disappearance constant (K) of galactose from the blood stream was calculated by the equation: $K = \frac{2.3\ (\log C_1\text{-}C_2)}{t_2\text{-}t_1}$, in which C_1 and C_2 are the initial and final

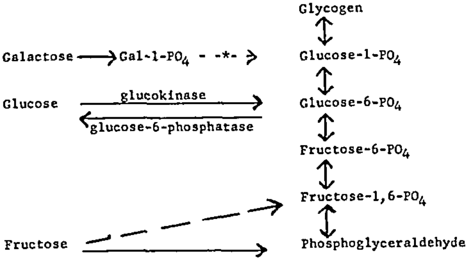

Fig 1—*Diagrammatical representation of the site of entry of galactose, glucose, and fructose into the hepatic glycolytic cycle* *Intervening reactions in the conversion of galactose-1-PO₄ to glucose-1-PO₄ are omitted.

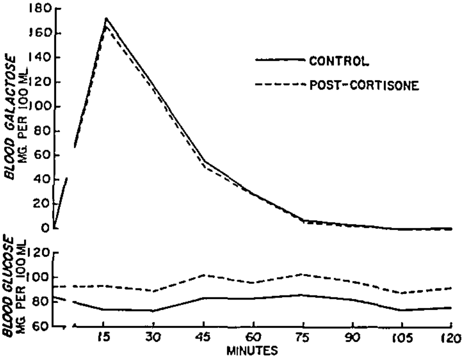

FIG. 2.—The effects of single oral dose cortisone administration on the mean galactose tolerance tests and associated blood glucose values in normal men The time in minutes after "O" refers to the time from the end of the infusion period

TABLE I. THE EFFECT OF A SINGLE ORAL DOSE OF CORTISONE ON THE GALACTOSE TOLERANCE TEST

	BLOOD GALACTOSE (MG. PER 100 ML.)													
	15 MIN.*		30 MIN.		45 MIN.		60 MIN.		75 MIN.		90 MIN.		120 MIN.	
SUBJECT	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE
1	221	*213*	165	*157*	85	*83*	33	*47*	21	*12*	11	*4*	7	*1*
2	176	*178*	144	*127*	69	*57*	43	*41*	10	*7*	6	*2*	4	*0*
3	221	*203*	150	*114*	78	*74*	48	*38*	12	*6*	8	*2*	1	*0*
4	194	*168*	126	*114*	73	*60*	41	*32*	13	*9*	7	*3*	0	*2*
5	138	*145*	103	*105*	51	*46*	31	*25*	6	*12*	6	*6*	1	*4*
6	217	*216*	151	*153*	86	*83*	50	*50*	10	*12*	1	*7*	0	*2*
7	176	*168*	126	*150*	58	*61*	32	*52*	1	*2*	0	*0*	0	*0*
8	127	*100*	79	*57*	22	*11*	8	*2*	2	*3*	2	*3*	2	*0*
9	153	*160*	85	*88*	37	*35*	17	*13*	6	*7*	5	*3*	1	*3*
10	136	*120*	62	*50*	26	*13*	6	*1*	6	*0*	3	*0*	4	*0*
11	146	*154*	85	*78*	35	*36*	13	*7*	1	*0*	0	*0*	0	*0*
Mean	173.2	*165.9*	116.0	*111.6*	56.4	*50.8*	29.3	*28.0*	8.0	*6.4*	4.5	*2.7*	1.8	*1.1*
P Value	> 0.1		> 0.1		< 0.01		> 0.1		> 0.1		> 0.1		> 0.1	

*Time after completing the intravenous infusion of 50% galactose.

TABLE II BLOOD GLUCOSE CONCENTRATION AFTER GALACTOSE INFUSION

SUBJECT	PRE INFUSION		BLOOD GLUCOSE (MG PER 100 ML)													
			15 MIN *		30 MIN		45 MIN		60 MIN		75 MIN		90 MIN		120 MIN	
	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE	CONTROL	CORTISONE
1	86	*97*	65	*75*	53	*71*	75	*94*	92	*100*	84	*119*	80	*110*	77	*99*
2	87	*108*	77	*93*	54	*95*	80	*119*	83	*113*	96	*127*	89	*121*	86	*115*
3	82	*84*	35	*55*	50	*53*	66	*68*	66	*64*	82	*86*	79	*85*	75	*86*
4	92	*101*	93	*112*	96	*110*	106	*108*	101	*110*	109	*110*	107	*108*	89	*101*
5	78	*82*	76	*99*	66	*89*	76	*85*	73	*85*	78	*91*	77	*86*	77	*89*
6	94	*87*	56	*59*	53	*56*	72	*82*	75	*82*	90	*95*	88	*90*	76	*74*
7	83	*92*	65	*85*	69	*69*	84	*99*	76	*80*	90	*111*	80	*94*	70	*85*
8	85	*98*	94	*128*	91	*118*	101	*127*	95	*119*	86	*106*	84	*98*	80	*91*
9	73	*81*	74	*81*	88	*93*	85	*94*	82	*89*	78	*85*	73	*88*	76	*90*
10	80	*91*	87	*132*	98	*122*	88	*134*	85	*117*	74	*108*	66	*99*	60	*93*
11	79	*86*	92	*105*	87	*105*	85	*108*	82	*102*	80	*96*	77	*91*	75	*88*
Mean	83.5	*91.5*	74.0	*93.1*	73.2	*89.2*	83.5	*101.6*	82.7	*96.5*	86.1	*103.1*	81.8	*97.3*	76.5	*91.9*
P Value	< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01		< 0.01	

*Time after completing the intravenous infusion of 50 per cent galactose concentration of galactose during the period beginning t_1 minutes and ending t_2 minutes after the injection,[5] a similar statistical comparison in the interval between 15 and 45 minutes after infusion, with and without cortisone, revealed a P value greater than 0.05.

It is thus apparent that cortisone administration does not lead to impaired galactose tolerance.

2. The mean preinfusion blood glucose levels were significantly higher after cortisone than on the control day, and remained higher during the two hours following galactose administration (Table II, Fig. 2).

3. In six subjects (Subjects 1, 2, 3, 5, 6, and 7) there was a decrease in blood glucose 30 minutes after the galactose was given on the control day (Table II). In four instances (Subjects 1, 2, 3, and 6) this decrease from the fasting level exceeded 30 mg. per 100 ml. Five of these six subjects also exhibited a decrease in blood glucose following galactose infusion on the day of cortisone administration. Following cortisone, the decline was less striking than on the control day in three instances (Subjects 1, 2, and 6), more apparent in one (Subject 7), and remained unaltered in one (Subject 3). Five subjects exhibited a rise in blood glucose after the control galactose infusion varying from 4 to 18 mg. per 100 ml. This rise was increased by 14 mg. per 100 ml. or more in three subjects (Subject 8, 10, and 11) after cortisone administration and remained essentially unchanged in two (Subjects 4 and 9).

4. The urinary excretion of sugar after galactose infusion was not influenced by cortisone administration (Table III).

TABLE III. URINARY HEXOSE EXCRETION FOLLOWING THE GALACTOSE INFUSION

SUBJECT	CONTROL (GM.)	CORTISONE (GM.)
1	6.3	7.4
2	2.6	4.7
3	5.2	6.0
4	7.0	6.1
5	4.2	3.8
6	7.4	8.5
7	5.0	4.8
8	4.2	4.3
9	5.6	5.3
10	4.3	4.2
11	4.7	4.2
Mean	5.1	5.4

DISCUSSION

The precise mechanisms by which cortisone administration results in impairment of glucose tolerance remains unclear. Both increased gluconeogenesis and decreased glucose utilization have been implicated.[6-12] Our previous observations that cortisone altered glucose tolerance without affecting fructose tolerance suggested a site of action of the steroid in the metabolism of glucose. Since the pathways for glucose and fructose metabolism in the liver become identical at the triose level, cortisone must have, as one site of action, some reaction prior to the splitting of six carbon molecules into three carbon fragments in the glycolytic cycle Other possibilities such as a specific cortisone effect on alternate pathways of glucose metabolism (hexose monophosphate shunt) or a selective cortisone effect on glucose disposal in the periphery seemed less likely explanations

Since galactose enters the Embden Meyerhof scheme in the liver at a different level than either glucose or fructose (Fig 1), the effects of cortisone on galactose tolerance may permit further definition of the site of steroid action in intermediary metabolism Thus if galactose and glucose tolerance had been impaired without impairment of fructose tolerance, the results would have suggested that the steroid acted somewhere above the three carbon levels and below glucose 6 phosphate To be sure, such observation could also be explained by a cortisone effect on two different sets of reactions such as the conversion of galactose to glucose 1 phosphate as well as the conversion of glucose to glucose 6 phosphate by the glucokinase reaction On the other hand, the observation that galactose tolerance was not impaired by cortisone administration is entirely consistent with an adrenocortical steroid affect on the glucokinase reaction alone Levine and Goldstein[13] observed that cortisone produced marked resistance to the effect of insulin on blood glucose in the intact animal, without inhibiting the action of insulin on galactose distribution after evisceration The authors suggested that these divergent results are consistent with adrenocortical inhibition of glucokinase activity

The physiologic significance, if any, of the lower mean 45 minute blood galactose value on the day cortisone was given remains doubtful Hein Sekula and Siedek[14] reported a more rapid disappearance of administered galactose from the blood after ACTH or cortisone in association with increased galactosuria In their studies the cortisone was administered intramuscularly immediately after the galactose was given and data for individual experiments are not provided

The decline in blood glucose after galactose administration to normal subjects has been observed before [15] [16] It is also known that children with congenital galactosemia often have hypoglycemia [16] [18] The fact that the three subjects with blood galactose levels over 200 mg per 100 ml experienced striking decreases in blood glucose of more than 30 mg per 100 ml suggested the possibility that a high blood galactose level might stimulate insulin action or increased hepatic uptake of glucose Such a concept is perhaps not unreasonable in view of the structural similarities of the glucose and galactose molecules, as emphasized by Levine,[19] [20] who described a similar behavior of the two hexoses in eviscerated animals in response to insulin administration It is of interest, however, that Subject 9 who did not exhibit hypoglycemia when his blood galactose level was 153 mg per 100 ml, did not undergo a decrease in his blood glucose level when on a subsequent occasion he received twice as much galactose and his blood galactose level exceeded 200 mg per 100 ml

CONCLUSIONS

1 The effects of a single oral dose of 200 mg of cortisone acetate upon the intravenous galactose tolerance tests were studied in 11 normal subjects

2. Cortisone did not impair galactose tolerance.

3. These observations are consistent with the hypothesis that the glucokinase reaction is a site of cortisone effect. This is not conclusively proved, nor is the possibility of other sites of action precluded.

4. Intravenous administration of galactose may result in a decrease in the blood glucose level. This observation remains unexplained.

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EFFECT OF FRUCTOSE FEEDING ON GLUCOSE TOLERANCE IN MAN

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HILL, Baker, and Chaikoff[1] have reported that normal rats fed an adequate diet containing fructose as the sole carbohydrate display a loss of glucose tolerance Hill and associates ascribed the diminished capacity of the fructose fed rat to utilize glucose to an impairment of the hepatic glucokinase system caused by protracted fructose feeding They found that the glucose tolerance curves in fructose fed rats resembled those observed in fasted rats, however, in fructose fed rats the liver was the only tissue in which an altered metabolic pattern could be demonstrated in vitro

Subsequently, Hill and Chaikoff[2] demonstrated that dogs fed a diet for one week containing fructose as the sole carbohydrate displayed an impairment of glucose tolerance similar to that observed in the fructose fed rat Galactose feeding did not produce such an effect in the dog

Craig, Miller, and Drucker[3] studied human subjects who had consumed a diet containing fructose as the sole carbohydrate for 3 days No alteration of tolerance to intravenously or orally administered glucose was observed after fructose feeding in these subjects, compared to a similar 3 day regimen in which glucose was the sole carbohydrate Their results suggested that, unlike the rat and the dog, liver glucokinase activity in man was not decreased by a chronically lowered concentration of glucose in the portal vein blood Van Itallie, Shull, and McCann[4] reported similar findings in human subjects fed fructose diets for 5 day periods

In view of the conflicting results obtained in experimental animals on the one hand, and human subjects on the other, it seemed desirable to explore further the effect of fructose feeding on glucose tolerance in man In particular, it appeared essential to obtain information about the relative roles of the liver and of the extrahepatic tissues in the disposal of ingested glucose after a period of fructose feeding This problem was approached by use of peripheral capillary venous glucose differences as indexes of extrahepatic glucose assimilation

The findings indicate that, in man, fructose feeding does not lead to any impairment of net glucose tolerance To the contrary, after ingestion of a fructose containing diet there seems to be a significant increase in the rate at

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*Postdoctoral fellow National Heart Institute National Institutes of Health Bethesda Md Present address Department of Biochemistry University of Cambridge Cambridge England which peripheral tissues remove glucose from blood. Evidence obtained by indirect methods suggests that fructose feeding in man (as in the rat and the dog) may temporarily diminish the rate of glucose uptake by the liver. However, this effect is masked in the conventional glucose tolerance test by the increased peripheral glucose uptake that seems to follow ingestion for 5 days of a diet in which fructose is the sole carbohydrate.

PROCEDURE

1. *Subjects.*—Eleven graduate students without evidence of disordered carbohydrate metabolism served as subjects for 19 experiments.

2. *Experiments.*—(a) In 5 subjects, glucose tolerance after ingestion of a self-selected diet containing at least 250 Gm. of carbohydrate per day for 3 days was compared with glucose tolerance after a 5-day period during which a diet was consumed that contained fructose as the sole carbohydrate. (b) In 4 subjects, glucose tolerance was determined after 3 days of carbohydrate deprivation and compared with glucose tolerance after ingestion of a self-selected diet containing at least 250 Gm. of carbohydrate per day. (c) In 6 subjects, combined glucose-epinephrine tolerance tests after fructose feeding were compared with similar tests performed after a normal diet. Preparatory diets and procedures were the same as in 2, a. (d) In one subject, an oral fructose tolerance test was performed before and after he had remained on the fructose diet for 5 days.

3. *Diets.*—

Fructose diet: This diet furnished at least 250 Gm. of fructose* per day. Protein and fat were provided by meat and eggs. Sugar-cured meats were not used. The caloric intake was adequate to maintain body weight. The fructose was consumed as a dilute aqueous solution in small quantities evenly distributed throughout the day.

Carbohydrate-free diet: This diet was identical with the "fructose diet" except that it contained virtually no carbohydrate (less than 1.0 Gm. per day).

Normal, self-selected diet: This diet provided 250 Gm. or more of carbohydrate per day for at least 3 days before each carbohydrate tolerance test was performed.[5]

4. *Carbohydrate Tolerance Tests.*—All subjects were studied in the morning approximately 14 hours after the previous meal. In experiments 2, a and 2, b, 100 Gm. of glucose dissolved in water was administered by mouth, and capillary (finger tip) and antecubital (venous) blood sugar levels were measured at half-hour intervals for 2 hours thereafter. Control blood sugar levels were also determined. (Capillary blood contains the same concentration of glucose as arterial blood.[6])

In experiment 2, c, 30 Gm. of glucose in aqueous solution was administered by mouth 10 minutes after subcutaneous injection of 0.004 mg. of synthetic epinephrine per kilogram of body weight. Blood sugar samples were obtained immediately prior to epinephrine injection and at 30, 45, 60, 75, and 90 minutes after ingestion of the glucose.

In experiment 2, d, 50 Gm. of fructose in aqueous solution was administered by mouth, and blood fructose and glucose values and serum inorganic phosphate levels were determined at 30-minute intervals for 2 hours.

5. *Methods.*—Glucose in whole blood was measured by the method of Somogyi[7] as modified by Nelson.[8] Fructose was measured by the method of Roe.[9] Serum inorganic phosphate was measured by the method of Fiske and Subbarow.[10] Venous and capillary blood samples were collected as previously described.[11] Relative assimilation indexes (R.A.I.) were calculated for each experiment in which capillary-venous glucose differences were measured, according to Somogyi's formula.[12] (Theoretic considerations underlying this type of calculation have been discussed by Hlad, Elrick, and Witten.[13])

$$\text{R.A.I.} = \frac{100 \times \Sigma(\text{C-V})}{\Sigma(\text{i})}$$

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*Kindly provided by Mead Johnson & Company, Evansville, Ind.

where, $\Sigma(C\ V)$ = sum of capillary venous differences
$\Sigma(i)$ = sum of the increments in capillary blood glucose over the control level during the corresponding time interval

Probabilities were estimated according to Student's test of "t".[14]

RESULTS

The mean glucose tolerance curves (venous blood) in the normal subjects on (a) "normal," (b) fructose containing, and (c) carbohydrate free diets are shown in Fig. 1. A striking impairment in glucose tolerance is evident in the subjects who were on a carbohydrate free diet for 3 days. When the mean glucose tolerance after 5 days of fructose feeding is compared with the mean glucose tolerance after a regular diet no such impairment is observed. If anything, glucose tolerances after fructose feeding are slightly improved as compared with those after a regular diet.

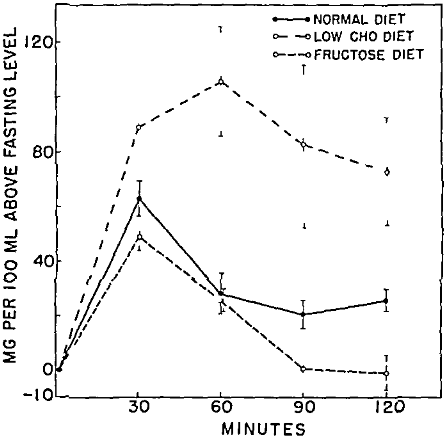

Fig. 1.—Effect of different diets on mean oral glucose tolerance (venous) of normal subjects. Vertical bars represent one standard error.

The relative (glucose) assimilation indexes (R.A.I.) calculated for the normal subjects on the 3 dietary schedules are given in Table I along with the individual capillary and venous blood sugar increments. The mean relative assimilation index of 63.3 ± 12.5* after the fructose diet is significantly greater ($p < 0.001$) than the mean relative assimilation index of 36.4 ± 6.7 after the regular diet. The mean relative assimilation index of 14.4 ± 10.3 after the carbohydrate free diet is significantly lower ($p < 0.01$) than that after the regular diet.

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*S.D. = $\sqrt{\frac{\Sigma(x-\bar{x})^2}{(n-1)}}$

TABLE I. EFFECT OF FRUCTOSE DIET AND LOW-CARBOHYDRATE DIET ON GLUCOSE TOLERANCE IN NORMAL SUBJECTS

SUBJECT	MINUTES AFTER INGESTION OF 100 GM. GLUCOSE								RELATIVE ASSIMILATION INDEX (R.A.I.)
	30		60		90		120		
	C*	V†	C*	V†	C*	V†	C*	V†	
	After Regular Diet								
T. R.	90	66	20	5	34	18	37	25	36.5
G. A.	59	45	48	29	25	3	31	16	42.9
R. C.	91	79	60	41	32	18	46	29	25.3
D. B.	91	83	68	26	27	14	28	19	32.2
R. S.	73	48	34	18	48	33	31	10	40.3
J. G.	90	59	89	50	63	39	42	20	41.1
					Mean and Standard Deviation				36.4 ± 6.7
	After Five Days on Fructose Diet								
T. R.	66	42	46	20	21	-3	22	-3	62.5
G. A.	78	53	39	13	23	-8	1	-25	79.4
R. C.	71	58	57	26	34	3	22	2	51.0
D. B.	59	35	59	41	32	11	34	3	51.6
R. S.	88	60	62	28	34	8	32	14	72.2
					Mean and Standard Deviation				63.3 ± 12.5
	After Three Days on Low-Carbohydrate Diet								
G. A.	109	80	106	73	91	70	78	46	30.4
R. C.	110	98	152	138	164	151	138	123	9.7
D. B.	122	95	87	67	65	51	39	33	20.4
J. G.	105	83	159	146	167	156	137	93	16.7
					Mean and Standard Deviation				14.4 ± 10.3

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.

In Fig. 2 the effect of fructose feeding on the response of normal subjects to the combined epinephrine-glucose tolerance test is shown. It is apparent that after fructose feeding a marked impairment in tolerance to glucose occurred during the first 45 minutes after glucose ingestion.

The individual capillary and venous blood sugar values for the same experiment are given in Table II as well as the relative assimilation indexes calculated for each individual tolerance test. These data show that, although the mean capillary and venous blood sugar values are much higher at the 30- and 45-minute intervals after fructose feeding than after the normal diet ($p < 0.01$ and $= 0.02$), the associated mean relative assimilation indexes are not significantly different.

To exclude the possibility that fructose feeding might have increased the liver glycogen content to a degree that the hyperglycemic response to epinephrine was enhanced, the response of the blood sugar to epinephrine administration was measured in three individuals before and after five days of fructose feeding. The mean hyperglycemic response after fructose feeding for these three subjects proved to be slightly lower after fructose feeding than it was following ingestion of a regular diet (Table III).

The effect of prolonged fructose feeding on the ability of the body to convert ingested fructose rapidly to glucose was determined in one subject (Fig. 3). No change in fructose tolerance or in the blood glucose response to fructose administration was observed following consumption of the fructose-containing diet of 5 days.

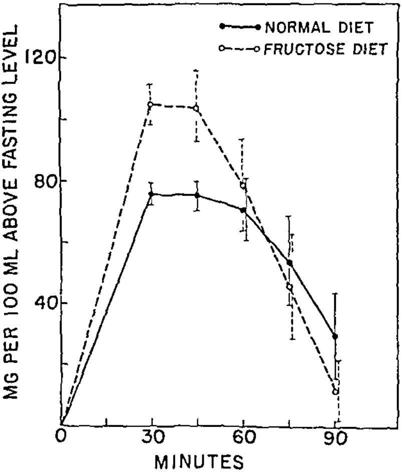

Fig 2—Mean hyperglycemic responses (venous) to oral glucose and subcutaneous epinephrine (glucose-epinephrine test) in normal subjects before and after fructose diet Vertical bars represent one standard error.

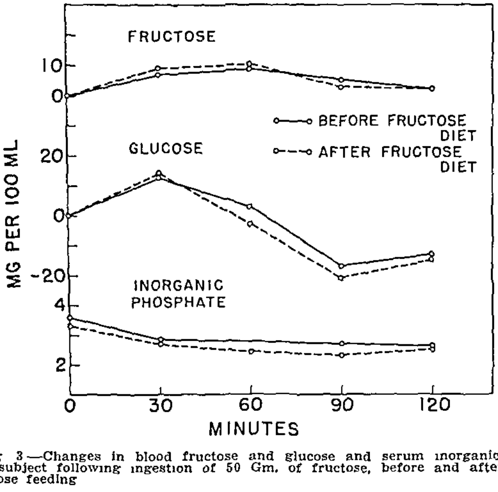

Fig 3—Changes in blood fructose and glucose and serum inorganic phosphate in a normal subject following ingestion of 50 Gm. of fructose, before and after a 5-day period of fructose feeding

TABLE II. EFFECT OF FRUCTOSE FEEDING ON HYPERGLYCEMIC RESPONSE TO GLUCOSE-EPINEPHRINE ADMINISTRATION IN NORMAL SUBJECTS

	MINUTES AFTER INGESTION OF 30 GM. GLUCOSE										RELATIVE ASSIMILATION INDEX (R.A.I.)
	30		45		60		75		90		
SUBJECT	c*	v†	c*	v†	c*	v†	c*	v†	c*	v†	
					After Regular Diet						
D. B.	82	69	76	66	69	65	51	44	18	15	12.5
L. B.	98	87	69	57	40	30	16	11	−6	−6	17.5
R. C.	88	86	85	79	80	75	63	60	46	43	5.2
K. M.	81	65	94	77	83	61	53	23	–	–	27.3
J. S.	73	73	91	85	101	94	95	101	–	–	3.6
T. T.‡	83	79	88	90	99	100	88	86	79	73	2.3
						Mean and Standard Deviation					11.4 ± 9.7
					After Five Days on Fructose Diet						
D. B.	100	77	92	73	80	68	64	47	47	37	21.1
L. B.	146	122	96	65	27	6	3	−8	−13	−19	35.9
R. C.	131	111	115	101	101	84	64	52	27	21	15.8
K. M.	125	105	137	123	118	95	–	–	–	–	9.7
J. S.	124	118	140	135	125	118	102	94	–	–	5.3
T. T.‡	102	98	136	126	126	100	–	–	–	–	11.0
						Mean and Standard Deviation					16.5 ± 12.8

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.
‡Subject took 75 Gm. glucose by mouth.

TABLE III. MEAN RISE IN CAPILLARY AND VENOUS BLOOD GLUCOSE AFTER INJECTION OF EPINEPHRINE IN THREE NORMAL SUBJECTS BEFORE AND AFTER FRUCTOSE FEEDING

	MINUTES									
	30		45		60		75		90	
DIET	c*	v†	c*	v†	c*	v†	c*	v†	c*	v†
Normal	22	19	27	25	23	29	18	19	14	14
Fructose	19	20	20	22	16	18	16	12	9	8

*Capillary glucose increments over control value, in milligrams per 100 ml.
†Venous glucose increments over control value, in milligrams per 100 ml.

DISCUSSION

The data clearly indicate that net tolerance to glucose after 5 days of fructose feeding is not impaired in normal subjects. This is in distinct contrast to results in the rat[1] and in the dog.[2] In both of these species marked impairment of glucose tolerance has been observed after periods of fructose feeding lasting 3 and 7 days, respectively.

When glucose tolerance after fructose feeding is considered in terms of antecubital capillary-venous glucose differences, it becomes apparent that, following fructose feeding, there is an increased uptake of glucose in the forearm as compared with forearm capillary-venous differences measured during control glucose tolerance studies. The mean relative assimilation index after the fructose diet is significantly greater than that after a normal diet adequate in unrefined carbohydrate. From these observations it is inferred that fructose feeding, in the subjects studied, enhanced the rate at which the peripheral tissues removed glucose from the blood.

The enhancing effect of fructose feeding on peripheral glucose uptake was unexpected in view of the findings of Hill and associates in animals, nevertheless, the results are too striking to ignore. The mechanism of this effect of fructose feeding remains unexplained.

Net glucose tolerance after fructose feeding improved to a lesser degree than might have been predicted on the basis of the markedly increased relative assimilation indexes measured following the fructose diet.[13] This discrepancy suggested that the fructose feeding might have had an effect on the liver that was obscured by the increased rate of uptake in the peripheral tissues.

To test this possibility, a modified oral glucose tolerance test involving ingestion of a small quantity of glucose in combination with subcutaneously administered epinephrine was employed. The epinephrine was given because of its inhibitory effect on peripheral glucose uptake.[11, 12, 15, 16] It was hoped that by reducing the part played by the extrahepatic tissues in determining the rate of glucose removal from the blood, a possible effect of fructose feeding on liver uptake of glucose would be disclosed.

When the epinephrine glucose tolerance test was done before and after the 5 day fructose regimen, a highly significant decrease in glucose tolerance was observed following the fructose diet. This decreased tolerance was not associated with a decreased relative assimilation index, in contrast to the relative assimilation indexes following carbohydrate deprivation. Since fructose is known to be readily converted to glycogen in the liver,[17] the possibility was considered that the decreased glucose tolerance after fructose feeding when the glucose epinephrine tolerance tests were done was due merely to an enhanced hyperglycemic response to the epinephrine. However, when the hyperglycemic responses to epinephrine alone before and after fructose feeding were compared, they did not differ (Table III).

It is difficult to understand why changes in net glucose tolerance after fructose feeding of the kind reported for the rat and the dog have not been demonstrable in human subjects. The proportion of liver weight to total body weight is approximately the same in all three species, hence, it does not seem possible to account for the observed differences by reference to relative liver size. It is recognized that rat, dog, and man differ somewhat in their characteristics of carbohydrate metabolism, however, not enough is known about the relative roles of liver and extrahepatic tissues in glucose disposal in the three species to explain why man should fail to show the same impairment of over all glucose tolerance after a fructose diet as the rat and the dog.

It is assumed that ingestion of single doses of fructose is not associated with appreciable changes in blood glucose in nondiabetic individuals. However, the suggestion has been made that prolonged fructose feeding is associated with an increased ability of the body to convert fructose to glucose so that the feeding of fructose will be associated in due course with considerable rises in blood glucose.[18] Accordingly, the effect of 5 days of fructose feeding on the blood glucose response to a large quantity of ingested fructose was determined. No changes in the blood fructose and glucose curves after ingestion of 50 Gm. of fructose took place as a result of the 5-day fructose diet. In both instances, the blood glucose level rose slightly at first and then fell below postabsorptive values following fructose ingestion. Similar responses of blood glucose to fructose administration have been reported by Miller and associates[19] and by Pletscher, Fahrländer, and Staub.[20]

The impaired glucose tolerance curves and decreased relative assimilation indexes associated with carbohydrate deprivation are consistent with the findings of other investigators suggesting that the "Hofmeister" effect is due principally to inhibition of glucose uptake in the periphery.[21] It should be pointed out that while fructose feeding and carbohydrate restriction both might be expected to reduce insulin requirements materially, fructose feeding in adequate amounts would not be associated with any deficiency of liver glycogen.[17] In contrast, acute carbohydrate deprivation leads to pronounced diminution of hepatic glycogen stores.[21] Thus, it may be that hepatic glycogen depletion is the sine qua non of starvation diabetes. If this difference in liver glycogen status is kept in mind, it is not surprising that the effect of fructose feeding on carbohydrate tolerance is entirely different from that of carbohydrate restriction. If anything, fructose feeding is associated with an increased rather than a decreased rate of glucose uptake in the extrahepatic area.

SUMMARY

The effect on glucose tolerance of an adequate diet containing fructose as the sole carbohydrate was determined in a series of normal subjects. Information about the relative roles of liver and extrahepatic tissues in the disposal of ingested glucose after a period of fructose feeding was obtained by use of peripheral capillary-venous glucose differences as indexes of rate of extraction of glucose by forearm tissues.

Although over-all glucose tolerance and rate of uptake of glucose by the forearm tissues were markedly diminished following ingestion of a carbohydrate-free diet for 3 days, the 5-day fructose diet did not induce any impairment of net tolerance to glucose or in rate of peripheral glucose uptake. Indeed, the rate of peripheral glucose removal was significantly increased following ingestion of the fructose diet.

The effect of fructose feeding upon glucose tolerance was also determined in subjects who had been given a small quantity of epinephrine subcutaneously just prior to the glucose tolerance tests, in an attempt to reduce the influence of the extrahepatic tissues upon rate of glucose removal from the blood. When epinephrine-glucose tolerance tests before and after the 5-day fructose regimen were compared, a significant decrease in net glucose tolerance was observed following the fructose diet. The decreased tolerance was not associated with a decreased rate of peripheral glucose assimilation, as measured by capillary-venous glucose differences, and this result suggested that fructose feeding may have impaired temporarily the ability of the liver to remove glucose from the circulation. Hyperglycemic responses to epinephrine alone measured before and after comparable periods of fructose feeding did not differ significantly.

It is concluded that the impaired glucose tolerance associated with carbohydrate deprivation is due principally to a decreased uptake of glucose in the extrahepatic tissues, and that the effect of fructose feeding on glucose tolerance is entirely different from that of carbohydrate restriction

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THE ACUTE RESPIRATORY EFFECTS OF CHLORPROMAZINE IN MAN

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CHLORPROMAZINE is being used extensively in the practice of medicine and psychiatry and as a preanesthetic medication. Because of this widespread use, it seemed desirable to have a quantitative estimation of the respiratory action of this drug in man and thereby an evaluation of one of its potential dangers. Dobkin and his associates,[1] from a study of both anesthetized and unanesthetized subjects, concluded that chlorpromazine caused central depression of respiration. Using a closed circuit apparatus, such as those in common use for the estimation of basal metabolism rate, these authors found that after intravenous administration of the drug the tidal volume was consistently decreased, but arterial blood O_2 and CO_2 contents were unchanged. Since a CO_2 absorber was in the system, no measurement of CO_2 output was made. Tenney[2] has emphasized that for accurate interpretation of drug effects on the respiratory mechanisms, a measure of alveolar ventilation, arterial CO_2 tension, and CO_2 output must be available.

The purpose of this communication is to report the effects observed in normal individuals and emphysematous patients after the administration of chlorpromazine.

SUBJECTS FOR STUDY

The age, physical characteristics, and diagnosis of each of the patients studied are recorded in Table I. Subjects 1 through 7 were patients admitted for a scheduled surgical procedure and by history, physical examination, and chest x ray were found to be normal except for the defect listed under diagnosis. These individuals are referred to below as normals. Subjects 8 and 9 had both had previous clinical and physiologic study with findings characteristic of chronic pulmonary emphysema. Subject 8 had recently recovered from right heart failure and CO_2 narcosis.

TABLE I. PHYSICAL CHARACTERISTICS AND DIAGNOSIS OF PATIENTS GIVEN CHLORPROMAZINE

PATIENT	AGE (YR.)	HEIGHT (CM.)	WEIGHT (KG.)	BSA (SQ. METER)	DIAGNOSIS
1 K. B.	33	178	80	1.98	Ventral hernia
2 L. R.	32	170	76	1.86	Pilonidal sinus
3 C. J.	45	176	66	1.80	Varicose veins
4 H. H.	30	178	74	1.92	Varicose veins
5 P. R.	49	174	77	1.92	Hemorrhoids
6 B. P.	37	173	73	1.86	Inguinal hernia
7 A. P.	32	179	79	1.98	Pilonidal sinus
8 L. G.	59	163	62	1.68	Emphysema
9 L. D.	65	166	68	1.76	Emphysema

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METHODS

Each patient was brought to the laboratory after an overnight fast and all observations were made while the individual was in a comfortable supine position An indwelling arterial needle was placed in the brachial artery and compressed air or 5 per cent CO_2 in air was administered through an open circuit using an anesthesia bag, mouthpiece, and a valve with a dead space of approximately 60 ml Expired air was collected and measured in a Tissot spirometer For each experiment, expired gas was collected for 3 minutes and arterial blood was sampled anaerobically during the middle minute The composition of inspired and expired gas was analyzed on the Scholander gas analyzer[3] The O_2 content and capacity and CO_2 content of the blood were determined by the method of Van Slyke and Neill[4] The O_2 and CO_2 tensions were measured by the technique of Riley, Proemmel, and Franke[5] In Subjects 3, 8, and 9, the CO_2 tensions recorded are those obtained by use of the Van Slyke and Sendroy charts[6] after whole blood pH had been determined on a Cambridge research model pH meter Five per cent CO in air was inspired prior to the 3 minute observation period for 12 minutes by Subjects 5 and 6, and for 25 minutes by Subjects 8 and 9 Blood pressure was measured by a sphygmomanometer

Volumes of ventilation were corrected to body temperature and pressure saturated with water vapor (BTPS), whereas quantities of gas exchanged are reported at zero degrees centigrade, 760 mm Hg pressure and dry (STPD) The respiratory dead space was calculated by use of the Bohr equation, utilizing the assumption that arterial is equal to alveolar CO_2 tension Alveolar ventilation was then calculated according to the formula alveolar ventilation = minute volume expired − (percentage dead space × minute volume expired)

The chlorpromazine used in these studies was Thorazine, 25 mg per cubic centimeter and the total dose administered was 25 mg intravenously or 50 mg intramuscularly When the intravenous route was used, the injection period was 5 minutes

RESULTS

The intravenous injection of chlorpromazine was followed in 15 to 30 minutes by a drowsy or sleeping state from which the patients could easily be aroused Only mild drowsiness occurred in one (Patient 8) who received the drug intramuscularly The results of measurements of ventilation, arterial blood gas composition, pulmonary gas exchange, blood pressure, and pulse are summarized in Tables II and III

A *Ventilatory Effects*—Following the intravenous administration of 0 32 to 0 38 mg per kilogram of chlorpromazine to 6 normal individuals (Subjects 1 to 6), there occurred only small decreases in total minute ventilation in 5 and in average tidal volume in 4 Respiratory frequency also showed small and variable changes except in Patient 2 whose rate decreased markedly However, it may be seen from the representative respiratory tracings reproduced in Figs 1 and 2 that irregularities of rhythm and tidal volume occurred These tracings are a record of the manner in which the Tissot spirometer was filled with expired air during the 3 minute periods Since spirometer volume was constant in inspiration, no record of the ventilatory pattern for this phase of respiration is shown Also, since the paper speed is slow, the record gives only a gross measure of the rate of air flow during expiration Nevertheless, the general pattern observed was one of regular shallow breaths with intermittent deep sighing ones This abnormality of ventilatory pattern led to an increase in physiologic dead space in all of these individuals and hence all

TABLE II. EFFECT OF CHLORPROMAZINE ON VENTILATION, ARTERIAL BLOOD GAS, AND PULMONARY GAS EXCHANGE

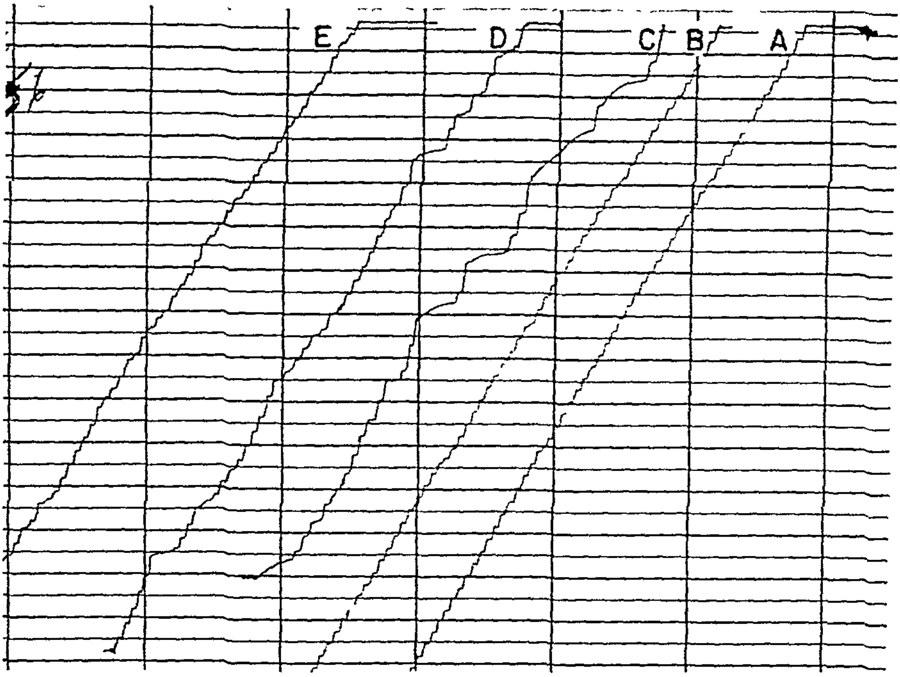
PATIENT	TIME (MIN.)	INSPIRED GAS	$\dot{V}_E$ (L./MIN.)	FREQ. RES.	V_T (C.C.)	Pa_{O_2} (MM. Hg)	Sa_{O_2} (%)	Pa_{CO_2} (MM. Hg)	$\dot{V}_{O_2}$ (C.C./MIN.)	$\dot{V}_{CO_2}$ (C.C./MIN.)	RES. QUOT.
A. Normal Individuals											
1. K. B.	-6*	Air	9.31	19	490	75	95	39	261	221	0.85
	0	0.32 mg. per kilogram chlorpromazine intravenously									
	+15	Air	6.80	17	400	74	93	40	263	153	0.58
	+45	Air	7.72	19	406	74	94	43	238	169	0.71
2. L. R.	-6*	Air	7.26	18.3	397	85	95	45	236	193	0.82
	0	0.33 mg. per kilogram chlorpromazine intravenously									
	+20	Air	5.93	9	659	79	94	54	223	163	0.73
	+40	Air	6.78	14.3	474	89	95	58	219	190	0.87
	+60	Air	6.85	16.3	420	93	95	47	200	172	0.86
3. C. J.	-6	Air	8.58	12	715	98	99	33	274	239	0.84
	0	0.38 mg. per kilogram chlorpromazine intravenously									
	+10	Air	8.41	16	526	--	95	38	270	194	0.72
	+20	Air	6.94	18.7	371	81	92	40	205	114	0.56
4. H. H.	-6*	Air	9.68	20	484	--	95	36	254	216	0.85
	0	0.34 mg. per kilogram chlorpromazine intravenously									
	+15	Air	10.67	21.3	501	--	94	38	246	214	0.87
	+30	Air	10.25	20	513	--	94	43	240	212	0.88
5. P. R.	-6*	Air	6.55	12.3	533	89	96	37	237	183	0.77
	0	0.33 mg. per kilogram chlorpromazine intravenously									
	+11	Air	6.26	14.7	426	--	96	37	207	152	0.73
	+18	Air	7.14	14	510	--	92	46	181	154	0.85
	+53	5% CO_2 in air	21.6	16	1,350	118	98	64	206	156	0.76

6 B. P.	-6*	Air	9 14	14 7	622	90	93	35	276	249	0 90
	0	0 35 mg per kilogram chlorpromazine intravenously									
	+10†	Air	9 26	16	579	81	92	37	284	245	0 86
	+30	Air	7 46	16 3	438	--	92	37	235	211	0 90
	+62	5% CO_2 in air	20 10	16 3	1,233	124	98	48	274	137	0 50
7 A P	-6*	Air	7 64	16 7	457	95	96	40	261	216	0 83
	0	0 63 mg per kilogram chlorpromazine intramuscularly									
	+30	Air	7 79	15 7	496	-	96	37	251	215	0 86
	+60	Air	7 89	17 3	456	99	94	37	243	215	0 88
		B Patients With Emphysema									
8 L G	-44	Air	8 28	21 7	382	39	68	59	210	163	0 78
	-3	5% CO in air	11 39	21	542	66	89	74	234	165	0 71
	0	0 8 mg per kilogram chlorpromazine intramuscularly									
	+21	Air	7 76	17 3	449	54	75	66	196	170	0 87
	+46	5% CO_2 in air	12 74	17 3	736	71	92	76	219	161	0 74
9 L D	-44*	Air	9 17	14 7	381	74	94	50	215	176	0 82
	-3	5% CO_2 in air	12 45	18	374	102	98	62	225	170	0 76
	0	0 69 mg per kilogram chlorpromazine intramuscularly									
	+60	Air	7 16	11	365	59	97	52	210	183	0 97
	+85	5% CO_2 in air	13 61	14 3	476	104	97	60	216	185	0 86

*Indicates two control studies

†Patient developed shock with unobtainable blood pressure and pulse at 15 minutes Responded to 2 c c of Neosynephrine intravenously

Key to abbreviations V_E = expired ventilation V_T = tidal volume Freq Res = frequency of respirations per minute PaO_2 = arterial oxygen tension SaO_2 = arterial oxygen saturation $PaCO_2$ = arterial carbon dioxide tension vO = oxygen uptake $\dot{V}_{CO_2}$ = carbon dioxide output Res Quot. = respiratory quotient.



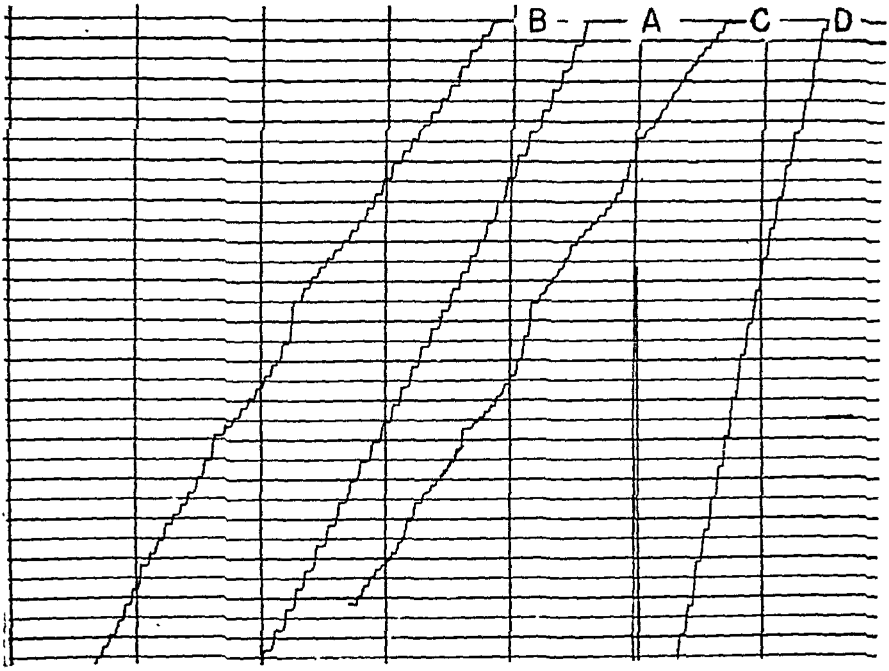


Fig. 1.—Spirometer tracing of respiratory excursions (Subject 2, L.R.). A and B = control. C = 20 minutes, D = 40 minutes, E = 60 minutes after I.V. chlorpromazine. Time = 1 minute between each vertical line. Inspiration = exactly horizontal since the spirometer volume is constant. Expiration = all lines with vertical component.

Fig. 2.—Spirometer tracing of respiratory excursions. A, B, and C = control, 10 and 20 minutes respectively, after I.V. chlorpromazine for Subject 3 (C.J.). D = inhalation of 5 per cent CO_2 62 minutes after I.V. chlorpromazine for Subject 6 (B.P.). See legend to Fig. 1.

showed a decrease in alveolar ventilation. These effects were at their maximum between 15 and 30 minutes after injection of the drug but were still present at the end of 60 minutes. They did not occur after a 0.63 mg. per kilogram dose given intramuscularly in a normal individual.

B. *Effects on Blood Gas Composition and Pulmonary Gas Exchange.*—Chlorpromazine given intravenously resulted in an elevation of arterial carbon dioxide tension of 6 mm. or more in 4 individuals (Subjects 2 through 5).

TABLE III. EFFECT OF CHLORPROMAZINE ON RESPIRATORY DEAD SPACE, ALVEOLAR VENTILATION, BLOOD PRESSURE AND PULSE

PATIENT	TIME (MIN.)	INSPIRED GAS	V_D/V_T (%)	V_A (L./MIN.)	B.P. (MM. HG)	PULSE
		A. Normal Individuals				
1. K. B.	−6*	Air	46	5.03	94/58	60
	0	0.32 mg. per kilogram chlorpromazine intravenously				
	+15	Air	50	3.40	98/54	80
	+45	Air	55	3.46	90/50	70
2. L. R.	−6*	Air	48	3.77	100/60	72
	0	0.33 mg. per kilogram chlorpromazine intravenously				
	+20	Air	55	2.66	100/60	76
	+40	Air	56	2.98	100/60	80
	+60	Air	53	3.22	80/40	72
3. C. J.	−6	Air	40	5.14	132/70	84
	0	0.38 mg. per kilogram chlorpromazine intravenously				
	+10	Air	46	4.55	120/70	100
	+20	Air	63	2.56	86/?	100
4. H. H.	6*	Air	45	5.32	100/60	68
	0	0.34 mg. per kilogram chlorpromazine intravenously				
	+15	Air	53	5.01	100/64	80
	+30	Air	57	4.42	100/60	80
5. P. R.	−6*	Air	36	4.19	124/68	76
	0	0.33 mg. per kilogram chlorpromazine intravenously				
	+11	Air	42	3.63	96/60	98
	+18	Air	58	2.99	75/56	60
	+53	5% CO_2 in air	77	4.97	100/60	90
6. B. P.	−6*	Air	30	6.40	114/64	70
	0	0.35 mg. per kilogram chlorpromazine intravenously				
	+10†	Air	37	5.84	108/60	80
	+30	Air	33	5.04	90/0	80
	+60	5% CO_2 in air	47	10.7	120/60	80
7. A. P.	−6*	Air	45	4.20	122/70	110
	0	0.63 mg. per kilogram chlorpromazine intramuscularly				
	+30	Air	34	5.14	122/70	110
	+60	Air	35	5.13	112/68	110
		B. Patients With Emphysema				
8. L. G.	−44*	Air	73	2.24	130/80	80
	−3	5% CO_2 in air	66	3.87		
	0	0.8 mg. per kilogram chlorpromazine intramuscularly				
	+21	Air	70	2.33	130/80	80
	+46	5% CO_2 in air	72	3.57		
9. L. D.	−44*	Air	61	3.58	130/80	80
	−3	5% CO_2 in air	54	5.73		
	0	0.69 mg. per kilogram chlorpromazine intramuscularly				
	+60	Air	56	3.15	130/80	80
	+85	5% CO_2 in air	50	6.81		

*Indicates two control studies.

†See footnote to Table II.

Key to abbreviations: V_D = physiological dead space; V_T = Tidal Volume; V_A = Alveolar Ventilation; B.P. = Blood Pressure.

its respiratory action is also a central depression. The type of respiration observed here was like that described by Hoff and Breckenridge[9] as one of the basic medullary rhythms which appears when the medulla and lower pons are released from suppressor impulses arising in the upper brain stem or cortex by decerebration or by narcotics and sleep. According to these authors, the medullary respiratory center has an intrinsic autorhythmicity which is modified by facilitatory and suppressor influences from numerous central and peripheral sources. The inhalation of 5 per cent CO_2 after chlorpromazine depression reesulted in a restoration of a regular rhythm and a normal increase in tidal volume yet the total ventilatory response was reduced. It thus seems possible that the drug has a depressant action upon the medullary respiratory center as well as more rostral areas of the brain.

Although the magnitude of the changes in blood gas tensions and contents were not great following the use of chlorpromazine, it should be pointed out that the techniques used in this study tend to obscure the greater deviations from normal that probably occurred. Since arterial blood was sampled during a one-minute period, the O_2 and CO_2 contents found represent a physiologic average of the net effects of many respiratory and cardiac cycles. In like manner, the values for alveolar ventilation, being derived from the respiratory volume and frequency of a 3-minute period, are also averages. Riley and Cournand[10] have pointed out that variations in the partial pressure of O_2 and CO_2 in alveolar gas and alveolar capillary blood from moment to moment must occur and that such variations during the respiratory cycle are likely to have an appreciable effect upon the physiologic dead space and venous admixture, particularly when the breathing is irregular. Although the calculated net effect of chlorpromazine was a reduction in alveolar ventilation, it seems likely in view of the irregularity of ventilatory depth that variations in ventilation-perfusion ratios with respect to time occurred and contributed to the gas exchange defects.

From the standpoint of therapeutic usage, these respiratory effects of chlorpromazine and the occurrence of shock in one patient emphasize the potential hazard of the intravenous route of administration. In addition, caution and close observation would seem to be advisable when this drug is administered by any route to patients who are to receive further central nervous system depressants or to individuals whose respiratory center is already depressed, such as patients with chronic pulmonary emphysema and CO_2 retention.

Diminished O_2 consumption following the use of chlorpromazine has been observed previously, both in vitro and in vivo, in animals and in man.[11] The mechanism of this action has not been elucidated.

SUMMARY AND CONCLUSIONS

The acute effects of chlorpromazine on ventilation, blood gas composition, and pulmonary gas exchange have been measured in 7 normal subjects and 2 patients with chronic pulmonary emphysema and CO^2 retention. The results

demonstrated were an irregularity of rhythm and amplitude of ventilation, alveolar hypoventilation, a decrease in O_2 uptake and CO_2 output and a diminished sensitivity of the respiratory mechanism to CO_2 inhalation These observations indicate that chlorpromazine has a respiratory depressant action

We wish to thank Mr John R Potrafka and Mrs Sandra C Penttila for their technical assistance

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THE INFLUENCE OF STREPTOCOCCAL TOXIN ON THE COURSE OF CANINE EXPERIMENTAL NEPHROSIS

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IN A previous communication we reported on the production of experimental nephrosis in dogs by intravenous administration of serum from rabbits sensitized to dog kidney.[1] The renal disease so produced was characterized by hypoproteinemia, with reduction of serum albumin, alpha-1 globulin and gamma globulin, and increase in alpha-2 and beta globulin. Hyperlipemia, elevation of blood urea, proteinuria, and some signs of fluid retention were among the salient features of the disease. After a certain period the animals appeared completely recovered and their blood and urinary constituents returned to normal.

In patients suffering from the nephrotic syndrome the clinical history not infrequently reveals association of the disease with septic sore throat. Since the experimental renal disease so closely resembles in its salient features the clinical condition in man designated by the term "nephrotic syndrome," we decided to investigate in animals the influence of administration of streptococcal toxin on the course of the nephrotic state induced in dogs by administration of nephrotoxic serum from rabbits sensitized to dog kidney. A number of investigators[2-6] have produced this renal disease in rats, rabbits, and occasionally puppies with some minor variations in the different species. We found the dog to be a very satisfactory animal for the experimental production of this syndrome.

METHODS

In the present study we used young immunized dogs in which the serum proteins, urinary proteins, serum cholesterol, and blood urea were quantitated before and after the administration of the nephrotoxic agents and their concentrations were followed until the animals recovered.

Healthy dogs were divided into three groups. One group was given nephrotoxic serum prepared, according to the procedure previously described,[1] from rabbits which had been sensitized against dog kidney. Exsanguination of the rabbits was done by cardiac puncture, which yielded a larger volume of blood than the previous procedure of cannulation of the femoral artery. A second group of dogs was given a streptococcal culture filtrate. This filtrate was prepared by filtering a 4 day culture in veal infusion broth of *Streptococcus pyogenes* (C203M) through a Mandler candle.[†] A third group was given a mixture of nephrotoxic serum and streptococcal culture filtrate. The total dose of each of the

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[†] Dr. F. R. Heilman prepared the filtrate for this study.

nephrotoxic serum and of the streptococcal culture filtrate was 4.4 ml. per kilogram of body weight divided into 9 doses and given 3 times daily over 3 days. In the dogs receiving the combination of nephrotoxic serum plus streptococcal toxin, 9 ml. of streptococcal culture filtrate were added to the total dose of nephrotoxic serum, making 1 ml. of filtrate in each of the 9 doses administered.

During the period of study the animals were housed in separate metabolism cages, and at specified intervals blood and urine samples were obtained. The urine was collected over 24 hour periods. The total proteins in serum and urine were determined by Kingsley's procedure. The electrophoretic pattern of the proteins in the serum and urine was determined by paper strips on the apparatus of Durrum and associates,[8] and quantitation of the paper strips was done by the technique of Grassmann and associates.[9] Several times during the study the Tiselius procedure was used for determination of the electrophoretic patterns of serum and urinary proteins and the patterns were compared with those obtained by the paper technique. The procedure of Zak and associates[10] was used for determination of total serum cholesterol, and that of Van Slyke and Cullen[11] for blood urea.

After establishment of control electrophoretic patterns and values for serum and urinary proteins and the total serum cholesterol and blood urea, the individual animals in each cage were given the nephrotoxic agents as indicated in an earlier paragraph. *One hour before receiving the nephrotoxic serum alone or in combination with streptococcal* culture filtrate, each of the dogs was given subcutaneously 25 mg. of tripelennamine hydrochloride.*

RESULTS

In this study the effects of the nephrotoxic serum, prepared from rabbits sensitized against dog kidney homogenate, were uniform and the features of the experimental disease were similar to those previously reported by Stickler and us.[1] The dogs herein reported which received the nephrotoxic serum alone in total amounts of 4.4 ml. per kilogram of body weight over a period of 3 days exhibited severe hypoproteinemia with reduction of serum albumin and alpha 1 and gamma globulin, and variable but usually some increase of the alpha 2 and beta globulin. These manifestations appeared on the first day after the first injection and persisted for some time thereafter (Fig. 1). Usually the peak effect appeared within 2 days after the last injection of the nephrotoxic agent. The serum cholesterol and blood urea were consistently elevated soon after the course of injections of nephrotoxic agents was completed.

The various blood proteins appeared in the urine and the proteinuria became most severe immediately after completion of the 3 day course of injections of nephrotoxic serum. Fig. 1 demonstrates the control levels and the comparative effects of the nephrotoxic agents on the serum and urinary proteins, on the serum cholesterol and on the blood urea for more than 40 days. With few exceptions the effects of injecting nephrotoxic serum alone were much like those of the combination of nephrotoxic serum and streptococcal culture filtrate (Fig. 1). The following were the exceptions. The proteinuria induced by the injection of nephrotoxic serum alone completely disappeared by the twentieth day after administration of nephrotoxic serum, while the proteinuria which followed the combination of nephrotoxic serum and streptococcal toxin lasted much longer. In these animals proteinuria persisted throughout the observation period, which lasted for 40 days after the last injection of nephrotoxic serum and streptococcal toxin (Fig. 1). The serum cholesterol and blood urea were slightly higher in the dogs that received the combination than in the dogs that received the nephrotoxic serum alone.

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*Pyribenzamine hydrochloride, Ciba Pharmaceutical Products, Inc., Summit, N. J.

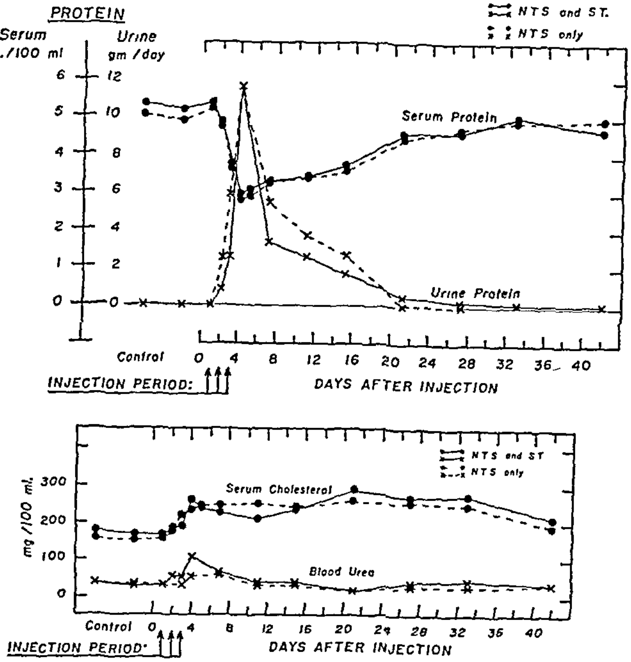
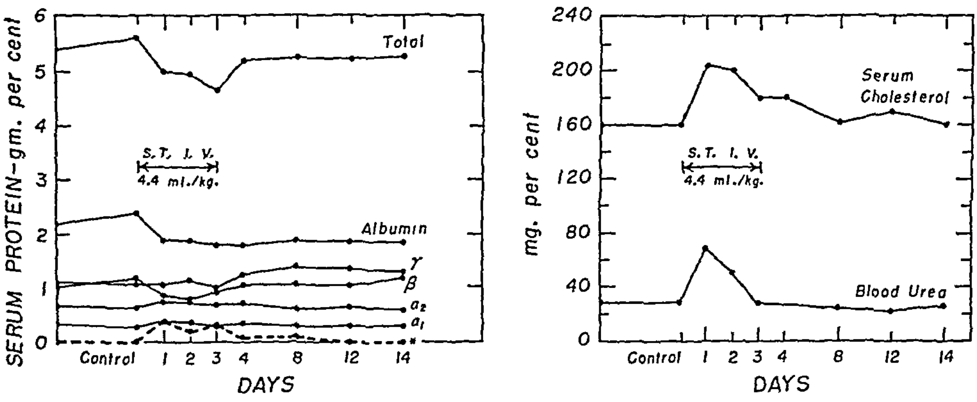


Fig. 1.—Comparative changes in certain constituents of blood and urine following intravenous administration of nephrotoxic serum (N.T.S.) and streptococcal toxin (S.T).



*Urine Protein gm./24 hours

Fig. 2.—Effects on dogs of intravenous administration of streptococcal toxin.

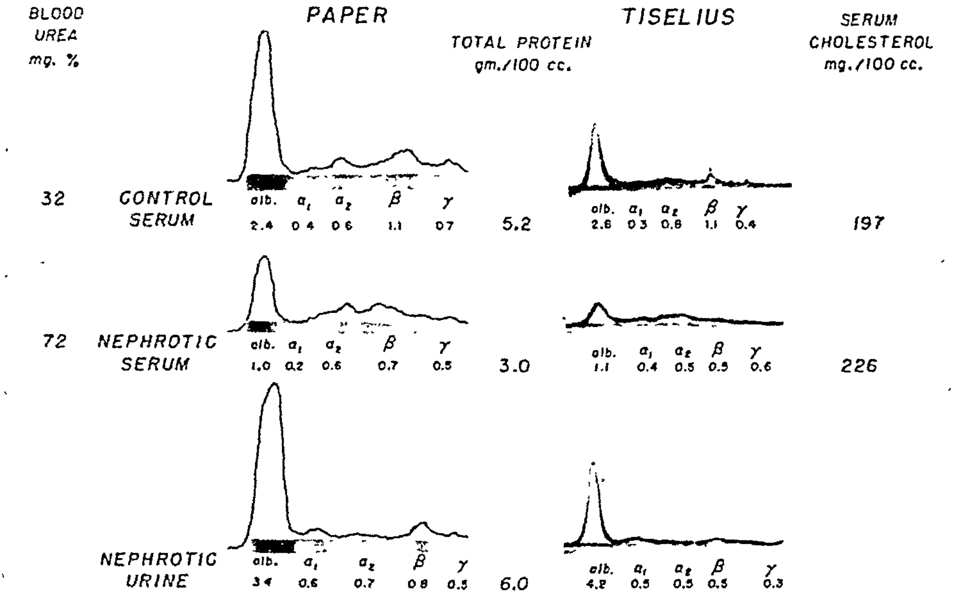
The administration of streptococcal culture filtrate alone, in total amounts of 4.4 ml. per kilogram of body weight given over a 3-day period, produced changes that were similar to those caused by nephrotoxic serum but were much milder and lasted for a much shorter duration. Fig. 2 demonstrates the various changes in the blood and urine following the intravenous administration of streptococcal culture filtrate alone. During and immediately after the period of administration of streptococcal toxin there occurred a definite but moderate reduction of total serum proteins and of serum albumin (Fig. 2). The serum cholesterol and blood urea were definitely elevated but for each the increase was transient (Fig. 2). Proteinuria occurred on the first day of injection of streptococcal toxin and lasted until the eighth day only; thereafter, no protein was found in the urine of the dogs which received streptococcal culture filtrate alone (Fig. 2). There was a definite reduction of the serum albumin with a definite increase of the blood urea and serum cholesterol, but the variations in the other fractions of the serum proteins were not consistent. In order to determine the effects of higher concentrations of streptococcal toxin, use of larger doses is planned in a future study.

TABLE I. INFLUENCE OF INTRAVENOUS ADMINISTRATION OF NEPHROTOXIC AGENTS ON CERTAIN CONSTITUENTS OF BLOOD AND URINE

CONSTITUENT	NEPHROTOXIC SERUM			STREPTOCOCCAL TOXIN			NEPHROTOXIC SERUM AND STREPTOCOCCAL TOXIN		
	CONTROL*	MAXIMAL CHANGE		CONTROL*	MAXIMAL CHANGE		CONTROL*	MAXIMAL CHANGE	
		AMOUNT*	PER CENT		AMOUNT*	PER CENT		AMOUNT*	PER CENT
Serum									
Total protein	5.00	−2.2	−44	5.53	−0.87	−16	5.30	−2.34	−44
Albumin	2.51	−1.65	−66	2.31	−0.51	−22	2.69	−1.83	−68
Alpha 1 globulin	0.36	−0.09	−25	0.32	−0.04	−13	0.34	−0.08	−24
Alpha 2 globulin	0.55	+0.09	+16	0.67	+0.09	+13	0.57	+0.30	+53
Beta globulin	1.03	+0.11	+11	1.11	+0.14	+13	1.07	−0.46	−43
Gamma globulin	0.61	−0.19	−31	1.10	−0.14	−13	0.63	−0.28	−44
Cholesterol (mg./100 ml.)	155	+103	+66	161	+43	+27	170	+118	+69
Blood urea (mg./100 ml.)	38	+59	+155	28	+40	+143	33	+75	+227
Urine protein (Gm./24 hr.)	0	11.6		0	0.32		0	11.6	

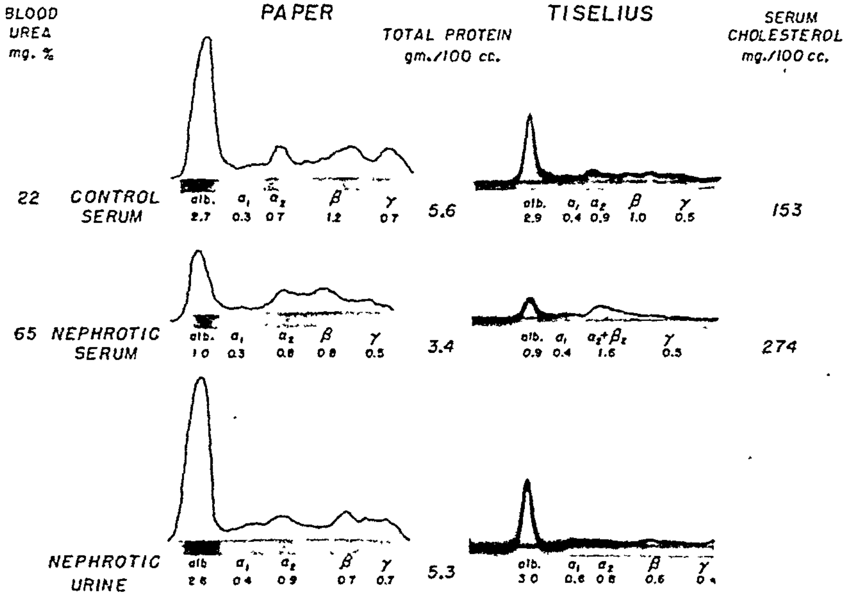
*Grams per 100 ml. unless otherwise indicated.

In our hands the paper procedure for the determination of the electrophoretic pattern of serum and urinary proteins worked very satisfactorily. In order to test the reliability of the paper procedure for electrophoresis, we applied the two procedures (paper electrophoresis and Tiselius electrophoresis) on the same specimens of serum and urine from the same animals before and after production of experimental nephrosis. Fig. 3 demonstrates the similarity of the findings by the two procedures, giving the various changes in blood and urinary proteins resulting from the nephrosis induced by the nephrotoxic agents used in this study. The curves obtained by the Tiselius and by the paper electrophoretic apparatus were much alike. The repeatability of such results and the close similarity of the findings by the two procedures gave us confidence in the paper procedure and made us conclude that it is reliable to use in the study of electrophoretic patterns of blood and urine.



Dog Given Nephrotoxic Serum I.V.

a



b

Dog Given Nephrotoxic Serum and Streptococcus Toxin I.V.

Fig. 3.—Electrophoretic patterns of the same samples of serum and urine determined by two different procedures (paper electrophoresis and Tiselius electrophoresis). *a*, Samples from dog given nephrotoxic serum intravenously. *b*, Samples from dog given nephrotoxic serum and streptococcal toxin intravenously.

Table I demonstrates the maximal changes produced by the nephrotoxic agents used in this study Not shown in the table is the fact that these maximal changes usually occurred on the first or second day after completion of the 3 day course of injections of the nephrotoxic agents The table gives in percentage the maximal changes from the control values that had been obtained before administration of the nephrotoxic agents Note that the combination of nephrotoxic serum and streptococcal toxin produced the greatest increase of blood urea (227 per cent) and of alpha 2 globulin (53 per cent), and also the greatest decrease of gamma globulin (-44 per cent) The association of gamma globulin with immunity reactions makes this reduction of gamma globulin in the serum a basic explanation for the susceptibility to infection encountered in such nephrotic states

SUMMARY

On healthy immunized young dogs a study was made of the influence of streptococcal toxin on the course of experimental nephrosis induced by the intravenous administration of serum obtained from rabbits sensitized to dog kidney After establishment of the control electrophoretic patterns and control values for serum proteins, serum cholesterol, blood urea, and urinary proteins the dogs were divided into 3 equal groups Group I was given rabbit nephrotoxic serum, Group II was given streptococcal culture filtrate, and Group III was given a mixture of rabbit nephrotoxic serum and streptococcal culture filtrate The total dose of each of the nephrotoxic serum and of the streptococcal culture filtrate was 4 4 ml per kilogram of animal body weight and was divided into 9 doses given intravenously over a period of 3 days Blood and urine samples were analyzed daily during the first week and 2 or 3 times a week thereafter for more than 40 days Nephrotoxic serum alone produced a uniform picture characterized by severe hypoproteinemia, with reduction of serum albumin, alpha 1 and gamma globulins and variable but usually some increase in the alpha 2 and beta globulins The serum cholesterol and blood urea were consistently elevated Proteinuria was severe but disappeared by the twentieth day after administration of nephrotoxic serum These manifestations were most marked immediately on completion of the course of injections of nephrotoxic serum

The intravenous administration of streptococcal culture filtrate alone produced changes which were similar to those caused by nephrotoxic serum but were very much milder and lasted for a much shorter duration However, intravenous administration of the combination of nephrotoxic serum and streptococcal toxin produced a picture much like that of nephrotoxic serum alone with the following exceptions the proteinuria which followed the use of the combination of nephrotoxic serum and streptococcal toxin persisted much longer (more than 40 days), and the reduction of gamma globulin was greater and the increase of serum cholesterol and blood urea was slightly higher than in the dogs which received nephrotoxic serum only

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THE PLASMA AMINO ACIDS IN PATIENTS WITH LIVER FAILURE

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IN 1849 Rokitansky[1] recognized crystals of tyrosine and leucine in the liver of patients with acute hepatic necrosis, but the importance of the liver in amino acid metabolism was not clearly revealed until 1924, when Bollman, Mann, and Magath[2] showed that the hepatectomized dog was unable to form urea from amino acids Although the total plasma amino acids are normal in mild liver disease, elevations are usually found in severe necrotizing hepatic disease[3]

The quantitative partition of the blood amino acids into individual components by column chromatography has only recently become possible[4, 5] Reports of the behavior of individual plasma amino acids in patients with liver disease[6, 8] or in experimental liver disease[9, 10] are limited This paper presents individual plasma amino acid levels in 2 normal subjects and in 7 patients with liver disease of varying severity, 5 of these patients were in liver coma The effects of intravenous sodium glutamate are noted in the 2 normal subjects and in 3 of the patients in hepatic coma

MATERIALS AND METHODS

Venous blood samples were collected in citrate or heparin The plasma was immediately separated by centrifugation, frozen, and stored at -18° C until used The plasma was deproteinized by ultrafiltration through "Visking" cellophane under nitrogen pressure in a refrigerated Monel metal apparatus[11] This apparatus separates approximately 70 per cent of the initial plasma volume as filtrate Chromatography was then performed on Dowex 50 columns according to the technique of Moore and Stein[4, 5] The amino acids were determined on the eluate by the colorimetric method of Stein and Moore[12] using a standard correction for each amino acid Glutamine is lost in varying degrees on the column, and glutamine, asparagine, and serine (G A S complex) come off together Glutamine was determined separately by enzyme hydrolysis with glutaminase derived from a rat kidney homogenate[13] The normal values by this method range from 350 to 760 μM/L Alpha ketoglutarate, pyruvate, and ammonia were determined by the methods of Seligson[14] Citric acid was determined by the method of Ettinger, Goldbaum, and Smith[15]

Pertinent information on patients used in this study is given in Table I Patients 1, 2, 5, 6, and 7 received an intravenous infusion of monosodium glutamate[*] All of these patients received a control infusion of glucose before and after sodium glutamate administration The glutamate was given in 5 per cent glucose at such a rate that the amount of glucose per unit time during this and the control periods remained constant Patients 1, 2, 5, 6, and 8 represent Patients 1, 2, 4, 5, and 10, respectively, in another paper[16]

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[*] The monosodium glutamate was generously provided by the Sherman Laboratories Detroit Mich

TABLE I. PERTINENT CLINICAL INFORMATION ON THE NORMAL SUBJECTS AND PATIENTS WITH CIRRHOSIS

NO.	AGE	SEX	DIAGNOSIS	DURATION (MONTHS)	ALBUMIN (GM./100 ML.)	BODY NUTRITION AND FOOD INTAKE	BILIRUBIN (MG./100 ML.)	MENTAL STATE	FACTOR PRECIPITATING COMA	OUTCOME
1	21	M	Normal	—	5.0	Good	0.5	Clear	—	—
2	26	M	Normal	—	4.6	Good	0.3	Clear	—	—
3	51	M	Mild cirrhosis; toxic hepatitis	0.5	2.6	Good Mild anorexia	7.0	Precoma	p-amino salicylic acid	Recovered
4	17	F	Wilson's disease; cirrhosis (severe)	12	1.4	Poor No intake, 6 days	14.0	Precoma Coma, 24 hr.	(?) Paracentesis	Died, 3 days
5	62	M	Cirrhosis (severe)	18	3.4	Poor No intake, 18 days	31.8	Coma, 24 hr.	Hemorrhage	Died, 3 days
6	44	M	Cirrhosis (severe)	0.5	2.8	Poor No intake, 10 days	19.0	Coma, 12 hr.	(?) Paraldehyde	Died, 12 days
7	46	M	Cirrhosis (severe)	4	2.2	Poor Mild anorexia	3.2	Coma, 6 hr.	Hemorrhage	Died, 1 day
8	52	M	Nonalcoholic cirrhosis (severe)	8	1.4	Poor No intake, 6 days	4.4	Coma, 24 hr.	(?) I.V. protein hydrolysate	Died, 15 days
9	12	M	Wilson's disease; cirrhosis (mild)	12	3.4	Good	0.6	Clear	—	Alive

RESULTS

All data are given in Table II Determination 1 A and 2 A were performed in normal subjects who were receiving infusions of glucose The values for each of the individual amino acids were within or below the range found in 17 normal subjects in whom plasma was obtained after a 6 hour fast Determination 9 A was in a patient with well compensated cirrhosis, the plasma amino acid pattern was almost normal—proline, glycine, histidine, and leucine concentrations were moderately depressed and tyrosine was increased twofold Determinations 3 A and B, and 4 A were in patients with severe liver disease in precoma In 3 A and B, although the total amino acid level seemed normal, taurine was elevated about twofold, and threonine, G A S complex, glycine, methionine, and histidine were mildly depressed In 4 A the total amino acid was definitely elevated but there were particular elevations in aspartic acid, threonine, methionine, tyrosine, phenylalanine, and lysine Patient 4, who was treated symptomatically, progressed into hepatic coma (at the time of 4 B) and died In determination 4 B, 36 hours after 4 A, the total amino acids increased threefold, but aspartic acid and glutamic acid fell Alanine, proline, cystine, and histidine showed the most marked rises (four to eightfold), taurine, G A S complex, glycine, methionine, tyrosine, phenylalanine, and lysine rose to lesser degrees

Determination 6 A was performed on a patient in light hepatic coma with underlying cirrhosis The total plasma amino acid level was markedly decreased, glutamic acid, proline, valine, methionine, isoleucine, tyrosine, phenylalanine, and histidine were particularly depressed

Determinations 4 B, 5 A, 7 A, and 8 A were performed in different patients in severe hepatic coma All 4 subjects showed increased levels of methionine (two to twentyfold) and tyrosine (two to fivefold) Three of the 4 subjects showed increased levels of phenylalanine (two to fivefold) and threonine (one and one half to sevenfold)

Two normal subjects and 3 patients with hepatic coma had plasma amino acids measured before and after an infusion of glutamate In the 2 normal subjects, the glutamic acid level rose to 1,700 and 1,130 μM/L, respectively, near the end of the infusion The glutamic acid level fell rapidly after completion of the infusion and, in Patient 1, returned to normal within two hours The level of glutamate fell to one third of the maximal value within 15 minutes in Patient 2 Both normal subjects showed a rise in alanine and in G A S complex, the latter persisting longer than the former Aspartic acid rose in the one subject in whom it was measured There were mild rises in other amino acids following glutamate infusion

In the comatose subjects (Patients 5, 6, and 8) the second determination was made immediately after the infusion of the glutamate, but only in Subject 6 did the glutamic acid level exceed that observed in the 2 normal subjects All 3 of the coma patients showed a rise in alanine (50 to 350 per cent of the control value) Two of the 3 patients showed a rise in G A S complex (40 and

TABLE

	NORMALS* ± S.D.	PATIENT 1† NORMAL A	PATIENT 1† NORMAL B	PATIENT 1† NORMAL C	PATIENT 2‡ NORMAL A	PATIENT 2‡ NORMAL B	PATIENT 2‡ NORMAL C	PATIENT 2‡ NORMAL D
μM of Amino acid N/L. plasma ultrafiltrate								
Taurine	48 ± 10	28	55	43				
Aspartic acid	24 ± 10	12	61	23				
Threonine	131 ± 20	79	91	80	79	78	73	76
Glutamine, asparagine, serine	498 ± 60	454	520	540	168	210	215	87
Glutamic acid	138 ± 80	91	1700	85	112	1130	775	400
Proline	230 ± 40	105	118	103				
Glycine	241 ± 20	153	155	147	82	100	89	57
Alanine	319 ± 40	285	408	282	200	305	235	182
Valine	230 ± 50	172	173	186				
Methionine	22 ± 5	25	20	23				
Isoleucine	75 ± 5	56	55	49				
Leucine	116 ± 5	107	117	106				
Tyrosine	60 ± 5	34	41	32				
Phenylalanine	71 ± 5	31	43	31				
Cystine	Trace	Trace	Trace	Trace				
Histidine	141 ± 20	121	46	72				
Lysine	161 ± 20	152	134	88				
Total**	2505 ± 400	1905	3737	1890				
μM/L. whole blood								
Ammonia (NH_3)	67.6 ± 10	166	72	52				
α-Ketoglutarate	13.0 ± 1.5	12.4	20.6	13.7	11.3	17.8	16.0	11.8
Pyruvate	73 ± 16	115	128	90	69.1	71.0	67.5	78.8
Citrate	79.2	70	58	82	80	110	127	109
Glucose mg./100 ml.		88	96	95	77	82	83	80
Glutamine		229	265					

*Amino acid normals were determined on 17 individuals. The ammonia normals were determined in 25 individuals. The pyruvate and alpha-ketoglutarate are based on 16 persons. See methods for citrate and glutamine.

†I-A, sample drawn at the end of glucose infusion and just prior to the glutamate infusion. 1-B drawn at end of glutamate infusion (24 Gm. in 195 min.). 1-C drawn 120 min. after 1-B.

‡2-A drawn at end of glucose infusion and just before glutamate infusion. 2-B after 200 min. of glutamate infusion (24 Gm. in 205 min.). Glutamate infusion continued for 5 min. after 2-B. 2-C drawn 5 min. after glutamate infusion ceased. 2-D drawn 15 min. after end of glutamate infusion.

§Specimen obtained 7 days after A.

200 per cent) and in aspartic acid (40 and 500 per cent). Two of the three had a rise in the total amino acid level of 2 and 8 times the control; the third subject had no significant change.

Citric acid, pyruvic acid, and α-ketoglutaric acid were elevated in the cases with severe liver failure. α-Ketoglutaric acid was the only one of the three that rose consistently following glutamate infusion.

DISCUSSION

Fig. 1 shows in a schematic fashion the principal tissues which affect the plasma level of amino acids. The liver plays a central role in amino acid metabolism, for it can release amino acids into the plasma; convert some amino acids to others; control, in part, the release of dietary amino acids into the peripheral blood stream; and remove amino acids from the plasma. Muscle and other tissues may also release amino acids into the blood stream or take them from the blood stream for the building of new protein or for energy. The

II

PATIENT 3 PRECOMA		PATIENT 4‖ COMA		PATIENT 5¶ COMA		PATIENT 6¶ COMA		PATIENT 7 COMA	PATIENT 8¶ COMA		PATIENT 9 CIRRHOSIS
A	B§	A	B	A	B	A	B	A	A	B	A
78	79	33	100	55	47	70	135	34	0	32	50
15	Trace	192	37	41	60	23	124	23	22	17	12
65	66	550	1083	182	226	53	111	204	14	151	116
295	230	920	2550	470	1030	144	85	1038	420	600	267
61	60	221	175	152	957	17	3730	129	157	191	140
208	111	240	1080	233	176	20	8	250	153	155	103
131	151	510	1410	366	387	87	135	300	286	262	132
243	220	430	3260	485	1490	75	280	410	230	324	297
138	67	150	373	130	182	20	49	166	137	98	146
13	Trace	225	534	41	52	Trace	Trace	105	45	47	37
46	76	66	168	60	106	6	Trace	50	27	32	37
75	119	110	313	82	173	27	42	106	53	71	67
77	61	130	349	126	262	Trace	8	175	169	138	173
87	43	124	438	185	148	3	12	188	90	60	67
Trace	Trace	Trace	350	Trace	Trace	Trace	Trace	5	Trace	Trace	12
42	101	182	830	229	170	5	5	308	60	92	32
147	95	455	1310	Not done		13	17	248	210	138	101
1721	1479	4538	14360	2837	5466	563	4761	3739	2256	2408	1789
60		57	172	140	88	148	102	320	191	158	82
39.8		57	43	30	144	53	76	37	24	19	13
148		175	620	156	260	222	106	355	122	108	75
235		230	276	73	122	128	114	368			117
88		114	177	93	122	91	92	53	208	178	83
				700	1240				1350	1440	

‖4 A precoma present for 12 hr. 4-B 36 hr after 4 A. Patient in deep coma for 24 hr.

¶5 A, 6-A and 8-A obtained before glutamate infusion was started and at the end of the glucose infusion. 5 B at the end of glutamate infusion (24 Gm in 2½ hrs). 6 B after 32 Gm in 4½ hr. 8 B after 20 Gm in 3½ hr.

**Total obtained from addition of the amino acids listed.

kidney, in addition, transforms certain amino acids into others (such as glutamine to glutamic acid) and can excrete amino acids. At present not all of the factors that lead to the excretion of amino acids by the kidney are known.[10, 17]

In Fig. 1, amino acids from absorption are shown as passing through the liver before reaching the plasma amino acid pool. This is not entirely correct because shunting of the blood around the liver often occurs in liver disease. Recent observations[18] indicate that significant amino acid absorption occurs by way of the thoracic duct, thus, in effect, a portion of the amino acids of absorption may be "shunted" to the systemic circulation in the normal.

In the data recorded in this paper, the rise in methionine and the rise in tyrosine were the most persistent of all of the individual changes of the plasma amino acids. Wu, Bollman, and Butt[7] found both of these amino acids elevated and, in addition, found glutamic acid, glutamine, and cystine high in liver coma. Walshe[6] reported a rise in methionine and glutamine and emphasized the diagnostic value of an elevated tyrosine in the urine in severe liver disease. Cachin, Durlach, and Blass[8] found that elevated tyrosine was the earliest change in the plasma in liver disease, and Schreier[19] emphasized the rise in plasma methionine in liver failure. The rise in both methionine and tyrosine in our patients is out of proportion to the rise in the total plasma amino acids. Methionine was depressed in several of the patients with milder liver failure, confirming the observation of Wu, Bollman, and Butt.[7]

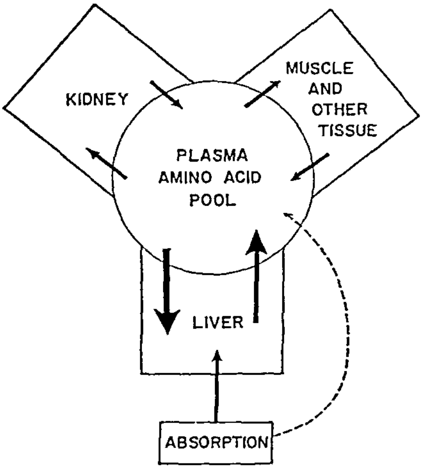

Fig. 1.—Schematic representation of the principal tissues that affect the plasma amino acid pool.

Four sources of the rise in plasma amino acids after severe liver damage seem possible: (1) dissolution of necrotic liver tissue,[20] (2) release from muscle or other tissue protein, (3) release from free amino acid pools in the tissues,[10] or (4) impaired utilization of plasma amino acids. Plasma amino acids rise at least as rapidly following hepatectomy as following severe liver necrosis[21] and both increases may be lessened by giving glucose.[10] The amino acid distribution of whole liver protein does not parallel the distribution of the rise in the plasma amino acids.[6] Dissolution of necrotic liver tissue does not, therefore, seem the source of the elevated amino acids. Flock, Mann, and Bollman[21] found that the rise in amino acids following hepatectomy occurred in the same proportion as in normal plasma, and they favored the theory that release of amino acids occurred from the free amino acid pool of muscle. In a later study,[22] however, they found that muscle and brain had a net increase in free amino acids; therefore some release from body protein had occurred. Of the possibilities, increased release of amino acids from muscle protein and impaired utilization by the liver are the most probable.

Many pathways of amino acid utilization occur in the liver, but of these information is available only on urea formation. Although urea formation has been found deficient in rare instances of liver failure,[23] it is usually normal.[24] Blood urea values were determined in Patients 4, 5, 7, 8, and 9 and were either normal or elevated. Altered energy production has often been observed in the presence of severe liver disease[25] and an elevation of the plasma amino acid level might be expected to correct this deficiency in part. The known actions of cortisone on protein metabolism would make the adrenal steroids possible mediators of this release of amino acids.

In animal studies, the increment above normal in plasma amino acids is similar in composition to the normal plasma amino acids and to amino acids of muscle tissue.[21] In the human being, glutamic acid, alanine, glycine, lysine, aspartic acid, proline, valine, isoleucine, and leucine are found in high molar concentration in whole muscle.[28] In liver failure, none of these show a rise in plasma out of proportion to the rise of the total plasma amino acids; lysine is a possible exception. If muscle protein were the source of the additional plasma amino acids, then there must be alterations in the subsequent metabolism of individual *amino acids to account for the lack of rise or the excessive rise of a* given amino acid.

The patients with mild liver failure did not show consistent abnormalities. The total amino acids seemed normal in all except Case 6, in which an extremely low value was *found. This is unexplained but may be a manifestation of poor* nutrition, for the subject had no oral intake for 10 days. Charkey, Kano, and Houghan[29] found a persistent fall in most of the individual plasma amino acid levels in normal subjects fasted for 48 hours. Patient 6 had inadequate calories for maintenance of body weight and no nitrogen intake for 10 days prior to study. However, Patient 5 underwent a comparable fast without depression of the total amino acids. Mellinkoff, Boyle and Frankland[30] found a more rapid disappearance of infused amino acids in mild liver disease than in normal persons. They suggested that decreased ability to mobilize or store glucose led to a higher output of insulin, which in turn accelerated the fall in amino acids. We have no data to support this concept.

Subjects 1 and 2 received sodium glutamate infusions and at the end of the infusion the levels were 1,700 and 1,300 μM/L, respectively. The dose used in this study (24 Gm.) would represent 2,300 μM/L in 55 liters, the approximate total body water of Patient 2, a man weighing 82 Kg. The plasma levels of this amino acid returned rapidly to normal after the end of the infusion. Associated with the fall in glutamic acid, the G.A.S. complex and alanine rose in both patients, and aspartic acid and leucine rose in the single patient in which they were measured. A rise in alanine out of proportion to the rise in other amino acids was found in all three of the coma patients treated with glutamic acid. Alanine most likely is formed as a result of the transamination reaction, in which one molecule each of glutamic acid and pyruvic acid combine to form one molecule each of alanine and α ketoglutarate. α Ketoglutarate rose after glutamate infusion in each patient. The rise in alanine and ketoglutarate following glutamate infusion was similar in normals and patients in liver coma.

There are several differences in the metabolism of amino acids in the patients with liver coma and the normal subjects. The total plasma amino acids are occasionally elevated in severe liver failure. Two of the five patients in hepatic coma showed significant elevations. The plasma concentration of methionine is disproportionately elevated in severe liver disease. A defect in plasma methionine in liver failure. The rise in both methionine and tyrosine in our patients is out of proportion to the rise in the total plasma amino acids. Methionine was depressed in several of the patients with milder liver failure, confirming the observation of Wu, Bollman, and Butt.[7]

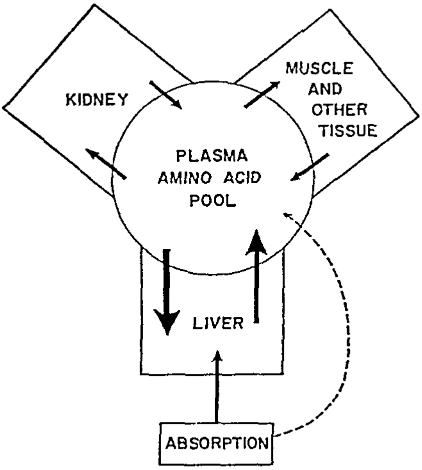

Fig. 1.—Schematic representation of the principal tissues that affect the plasma amino acid pool.

Four sources of the rise in plasma amino acids after severe liver damage seem possible: (1) dissolution of necrotic liver tissue,[20] (2) release from muscle or other tissue protein, (3) release from free amino acid pools in the tissues,[10] or (4) impaired utilization of plasma amino acids. Plasma amino acids rise at least as rapidly following hepatectomy as following severe liver necrosis[21] and both increases may be lessened by giving glucose.[10] The amino acid distribution of whole liver protein does not parallel the distribution of the rise in the plasma amino acids.[6] Dissolution of necrotic liver tissue does not, therefore, seem the source of the elevated amino acids. Flock, Mann, and Bollman[21] found that the rise in amino acids following hepatectomy occurred in the same proportion as in normal plasma, and they favored the theory that release of amino acids occurred from the free amino acid pool of muscle. In a later study,[22] however, they found that muscle and brain had a net increase in free amino acids; therefore some release from body protein had occurred. Of the possibilities, increased release of amino acids from muscle protein and impaired utilization by the liver are the most probable.

Many pathways of amino acid utilization occur in the liver, but of these information is available only on urea formation. Although urea formation has been found deficient in rare instances of liver failure,[23] it is usually normal.[24] Blood urea values were determined in Patients 4, 5, 7, 8, and 9 and were either normal or elevated. Altered energy production has often been observed in the presence of severe liver disease[25] and an elevation of the plasma amino acid

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EFFECT ON PORTAL BLOOD AMMONIUM CONCENTRATION OF ADMINISTERING METHIONINE TO PATIENTS WITH HEPATIC CIRRHOSIS

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IN CERTAIN patients with hepatic cirrhosis or Eck fistula, the administration of ammonium salts, protein, amino acids, or urea may induce impending hepatic coma, a syndrome characterized by mental confusion and a typical flapping tremor and usually associated with increased blood ammonium concentrations.[1-6] These nitrogenous substances may produce increased blood ammonium concentrations in similar patients without inducing the above untoward clinical signs.[5, 7, 8] Confusion also may follow methionine ingestion by patients with hepatic disease.[9-11] However, metabolites of methionine other than ammonium have been implicated by some investigators because blood ammonium concentrations did not change significantly in methionine-fed patients with cirrhosis, although some exhibited neurological deterioration. These metabolites were presumably formed by gastrointestinal bacterial action on methionine because methionine-induced neurological deterioration was prevented by chlortetracycline administration.[12]

In the present study, methionine as a source of portal blood ammonium was evaluated by determining arterial and portal collateral venous blood ammonium concentrations before and after methionine ingestion by patients with cirrhosis. Increases in portal collateral blood ammonium concentrations were observed after ingestion of this amino acid. Consistently significant increases were not observed after methionine ingestion when the same patients were pretreated with neomycin sulfate by the oral route.

MATERIALS AND METHODS

Three hospitalized male patients with histories of chronic alcoholism associated with poor food intake were studied. Clinical, laboratory, and histologic evidence indicated diagnoses of Laennec's cirrhosis. No patient had confusion, coma, tremor, shock, hemorrhage, or blood urea nitrogen concentrations above 15 mg. per 100 ml. (Table I).

The daily food of these patients was estimated to contain 30 to 90 Gm. of protein and approximately 250 mg. of sodium; there were no substantial dietary alterations during the observation period. Patients did not receive foods or medications containing nitrogen for at least 12 hours before each study was initiated.

Each study was performed by obtaining blood samples for ammonium-nitrogen determinations within two minutes of each other from a superficial abdominal vein and a brachial

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artery at intervals before and after the ingestion of 10 Gm. of DL methionine* in 250 ml. of water. Two studies were carried out for each patient. On the afternoon of the day following their first study, they were given neomycin sulfate† orally, 2 Gm. initially, followed by 1 or 2 Gm. every 4 to 6 hours, until the next morning when the second study was done.

Ammonium nitrogen (NH_4 N)‡ in blood and in the administered methionine solution was determined by a modified Conway technique.[6, 13] Blood was injected promptly into the Conway unit and a diffusion time of 10 minutes was employed. Results were calculated from the NH_4 N recovered from a standard as recommended for blood by Conway. Differences between NH_4 N concentrations in two consecutive blood samples drawn during the same day from the same site in a given fasting patient with cirrhosis ranged from 0 to 23 mcg. per 100 ml. This reproducibility held for superficial abdominal venous blood only if the blood *was drawn repeatedly from the same location in the same abdominal vein. Accordingly,* multiple sampling of abdominal venous blood was done in this manner.[8]

The 10 Gm. of methionine yielded 0.1 mg. of NH_4 N on analysis.

Serum bilirubin and blood urea nitrogen concentrations were determined by standard techniques.[14, 15] Bromsulphalein was determined in serum from blood obtained 45 minutes after the injection of 5 mg. of dye per kilogram of body weight.[16]

TABLE I. CLINICAL AND LABORATORY DATA IN THREE MALE PATIENTS WITH HEPATIC CIRRHOSIS*

PATIENT AGE	PHYSICAL EXAMINATION†	SERUM TOTAL BILIRUBIN (MG./100 ML.)	SERUM BSP (PER CENT RETENTION)	BLOOD UREA N (MG./100 ML.)
A. I. 58	Angiomata, hepatomegaly, splenomegaly	2.0	38	13
J. K. 49	Icterus, angiomata, hepatomegaly, edema	3.0	53	12
E. R. 55	Edema	1.8	38	12

*Diagnoses of Laennec's cirrhosis were confirmed by biopsy.

†All three patients had ascites and dilated superficial abdominal veins.

RESULTS

Before neomycin sulfate administration the three patients studied demonstrated significant increases in portal collateral blood NH_4 N concentrations (> 23 mcg. NH_4 N per 100 ml.) within an hour after methionine ingestion (Figs. 1 to 3). However, after 6 to 12 Gm. of neomycin sulfate had been given over *a period of 16 to 18 hours methionine did not produce significant increases* in portal collateral blood NH_4 N concentrations in two patients and produced only a minimal increase in the third. Except in one instance (Fig. 3) arterial blood NH_4 N concentrations did not increase significantly after methionine was ingested either before or after neomycin sulfate administration. Fasting portal collateral and arterial blood NH_4 N concentrations were lower after neomycin sulfate administration in 2 of the 3 patients (Figs. 1 and 3). The significance of this finding was difficult to evaluate because day to day variations in a given fasting patient with cirrhosis can be of this magnitude.

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*Kindly provided by Merck & Company, Rahway, N. J.

†Mycifradin sulfate, The Upjohn Company, Kalamazoo, Mich.

‡Whether ammonium normally is present in circulating blood or whether it is formed only after blood is shed has not been established. Volatile base released from blood upon addition of alkali is assumed to be ammonia.

The superficial abdominal veins employed contained portal blood to a measurable extent as evidenced by the following: Ammonium-nitrogen concentrations in blood obtained from these veins increased significantly and were in excess of increases in arterial blood NH_4-N concentrations in two of the neomycin-treated patients when NH_4Cl was given orally (50 mg. of NH_4-N to J. K. and 100 mg. to E. R.). Similar results were noted when 4 Gm. of L-glutamine were given orally to the third patient (A. I.) who had also received neomycin sulfate. Previous studies have demonstrated that glutamine can cause increased portal collateral blood NH_4-N concentrations in patients treated with neomycin.[8]

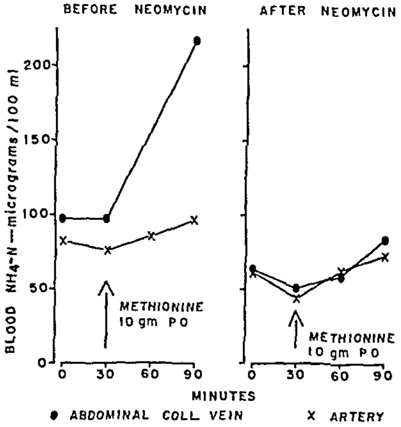

Fig. 1.—Effect of DL-methionine given orally. Patient J. K. with cirrhosis.

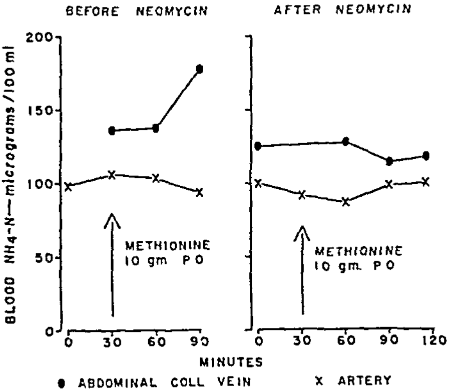

Fig. 2.

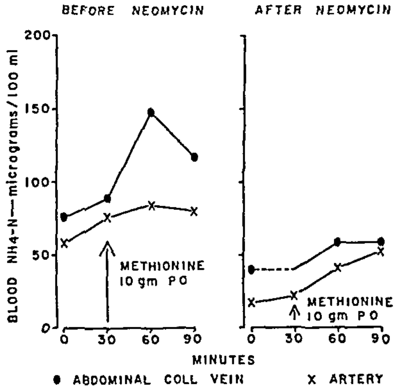

Fig. 3.

Fig. 2.—Effect of DL-methionine given orally. Patient E. R. with cirrhosis.
Fig. 3.—Effect of DL-methionine given orally. Patient A. I. with cirrhosis.

DISCUSSION

Increased concentrations of volatile base (NH_4-N) in portal collateral blood were noted shortly after each of three patients with hepatic cirrhosis ingested 10 Gm. of DL-methionine.

These increases in portal blood NH_4 N were probably not attributable to the NH_4 N found by analysis in the 10 Gm quantity of methionine administered (0 1 mg), because 20 mg of NH_4 N given orally in the form of an ammonium salt did not produce a portal collateral increase in other patients with cirrhosis[8] Further evidence for this hypothesis is that insignificant increases in portal collateral NH_4 N concentrations followed methionine administration to neomycin treated patients Increases from free NH_4 N that might have been contained in methionine would not have been modified by neomycin[8] This suggests that NH_4 N in detectable quantities was released only after methionine was metabolized

The fact that no significant increases in portal collateral blood NH_4 N concentrations followed oral administration of neomycin suggests that formation of the NH_4 N from methionine depended, in part at least, upon gastrointestinal bacterial action For example methionine might be deaminated by gastrointestinal bacteria or it could be absorbed and changed in part to urea which upon entering the gastrointestinal tract, might be deaminated by bacterial urease In either situation, increased portal collateral blood NH_4 N concentrations would result if the bacterial flora were not grossly altered

The increased portal collateral blood NH_4 N concentrations found here were at variance with other reported results, indicating that blood NH_4 N concentrations were not influenced by ingested methionine[12] However, the preparations of DL methionine employed, the dosages of amino acid given, the sites from which blood was taken, and the time intervals of blood sampling were not comparable

Whether or not the ability of methionine to induce impending hepatic coma is related to its ability to induce increased portal collateral blood NH_4 N concentrations is not known Other investigators have reported that chlortetracycline *may prevent the exacerbation of impending hepatic coma in methionine* fed patients with cirrhosis, presumably by its bacteriostatic effect[12] In this study, neomycin sulfate, an essentially nonabsorbable antibiotic, prevented increased portal collateral blood NH_4 N concentrations after methionine ingestion, apparently by altering the gastrointestinal flora concerned with ammonium production These observations are consistent with the hypothesis that ammonium or a related substance may play a role in methionine induced hepatic coma However, the possibility of a deleterious effect resulting from other bacteriologically produced metabolites of methionine cannot be eliminated

Speculations upon the quantitative aspects of gastrointestinal "ammonium" production from methionine contained in protein appear relevant After ingestion, 20 to 50 Gm of animal protein, containing an estimated 0 7 to 1 8 Gm of methionine,* produced increased arterial as well as portal collateral blood NH_4 N concentrations in some patients with cirrhosis[8] However, in the present study, 10 Gm of free methionine or about 6 times as much as contained in the above protein meals did not produce a significant increase in arterial blood NH_4 N concentrations except in one instance Moreover, portal blood NH_4 N increases after ingested protein, unlike those after methionine, were not modified significantly by prior administration of neomycin.[8] Thus, it appeared unlikely that the quantity of methionine contained in these protein meals accounted for much of the portal blood NH_4-N increase observed after their ingestion. However, in patients with severe liver disease, methionine given repeatedly in quantities sometimes employed therapeutically or as dietary supplements might not only produce increased blood "ammonium" concentrations but also induce the syndrome of impending hepatic coma.

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*Approximately 3 5 per cent of animal protein is methionine[17]

SUMMARY

Ten grams of ingested DL-methionine caused increased concentrations of ammonium or a related substance in the portal blood of each of three fasting patients with hepatic cirrhosis. These increases were largely prevented when each patient received 6 to 12 Gm. of neomycin sulfate orally during a period of 16 to 18 hours before methionine ingestion. These observations indicate that "ammonium" is formed from methionine or one of its metabolites and is absorbed into the portal blood. Furthermore, they suggest that the formation of "ammonium" from methionine is dependent to some extent upon the integrity of the gastrointestinal flora.

Methionine possibly may act like other nitrogenous substances containing or liberating "ammonium" which, like this amino acid, have induced the syndrome of impending hepatic coma in susceptible patients with liver disease or Eck fistula.

The technical assistance of Mrs. Helen Grodeck is gratefully acknowledged.

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LEUKOCYTE PHOSPHATASE ACTIVITY IN PREGNANCY

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THE alkaline phosphatase activity of circulating leukocytes may vary considerably. Marked increases in enzyme activity have been observed in several pathologic states including infection, trauma, hemorrhage, and pulmonary and myocardial infarctions, while in myeloid leukemia the enzyme activity is depressed.[1-3] The administration of corticotropin or cortisone to normal individuals results in considerable augmentation of leukocyte alkaline phosphatase activity.[2] The acid phosphatase activity of leukocytes, however, does not appear to vary appreciably from normal in these several conditions.[1, 2]

This report concerns the effects of normal pregnancy with its associated hormonal and hematological alterations on leukocyte alkaline and acid phosphatase activity.

MATERIAL AND METHODS

Leukocytes were obtained and their alkaline and acid phosphatase activities measured following the procedures of Valentine and Beck.[1] It was not necessary to add fibrinogen solution to blood from most women in the latter half of pregnancy since the sedimentation rate was quite rapid, undoubtedly due to the hyperfibrinogenemia of normal pregnancy. White blood cell counts were done in duplicate.

Leukocyte alkaline and acid phosphatase activities and white blood cell counts were determined in 12 healthy nonpregnant women, and in 81 apparently normal women during various stages of pregnancy and the puerperium. Of these 81 women, 10 were in the first trimester of pregnancy, 19 were in the second trimester, 14 were in the third trimester, 20 were at or near term and had just completed or were very near the end of labor, 8 were 3 days post partum, and 10 were 6 to 7 weeks post partum. In 15 instances fetal leukocytes were also obtained from cord blood at delivery simultaneously with maternal leukocytes and their phosphatase activities measured. In each of the 8 women during labor and the early post-partum period, repeated studies of leukocyte phosphatase activities were made during the first week of the puerperium. Also measured were enzymatic activities of leukocytes from 2 subjects with a fetus known to be dead in utero for several weeks, but in whom the placenta probably still retained some functional activity. Enzyme activities are expressed as milligrams of phosphorus hydrolyzed by 10 billion leukocytes in one hour.

RESULTS

The average leukocyte alkaline phosphatase activity in the normal nonpregnant females was 25.6 mg., quite similar to the values of 25 mg. found by

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Valentine[1, 2] and 23 1 mg reported by Wiltshaw and Moloney,[3] while acid pho phatase activity averaged 27 6 mg, differing slightly from the mean of 21 mg determined by Valentine

Fig 1 shows the increases in leukocyte alkaline phosphatase activity ass ciated with pregnancy During the first trimester the mean activity was 60 mg and rose to 75 0 mg during the second trimester The average activit during the third trimester but prior to the onset of labor was 76 5 mg Durin labor at or within one hour of delivery the mean leukocyte alkaline phosphata activity was 116 mg while the activity of leukocytes from cord blood at deliver was 76 0 mg Three days post partum the maternal mean leukocyte alkalin phosphatase activity increased to 213 mg Six weeks after delivery it average only 35 8 mg

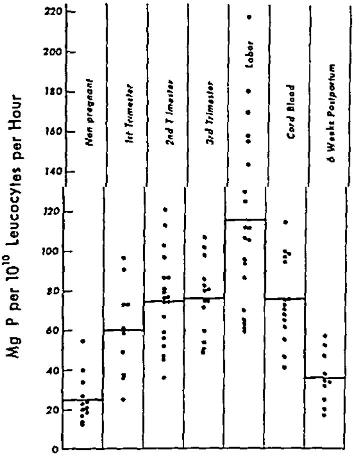

Fig 1—The individual and mean values for leukocyte alkaline phosphatase activit expressed as milligrams of phosphorus hydrolyzed by 10 billion leukocytes per hour are plotte for normal subjects during the nonpregnant state first trimester second trimester third tr mester at the end of labor for cord blood and for subjects 6 to 7 weeks post partum

Leukocyte acid phosphatase activity varied little during pregnancy an the puerperium, the mean values were first trimester 26 0, second trimester 26 2 third trimester 29 1, cord blood 23 8, 3 days post partum 27 1, and 6 to weeks post partum 26 0 mg of phosphorus liberated per hour by 10 billio leukocytes

The average concentration of leukocytes per cubic millimeter during thes periods were normal nonpregnant 7,790, first trimester 7,840, second trimeste 11,350, third trimester 9 030, labor 14,100, cord blood 18,350, 3 days pos partum 13,430, and 6 to 7 weeks post partum 6,480 There was no consisten relationship between the degree of elevation of the leukocyte alkaline phosphatase activity and the blood leukocyte concentration within any of these groups.

During the immediate post-partum period following labor the leukocyte alkaline phosphatase activity rose considerably, but the degree of elevation was quite variable, ranging from 124 to 410 and averaging 213 mg. 72 hours after delivery. In some instances the post-partum enzymatic activity increased markedly but the rise was not associated with an appreciable increase in the concentration of circulating leukocytes; in other instances the opposite was true. Data from 2 representative studies are shown in Fig. 2, *A* and *B*. In Fig. 2, *A*, a normal vaginal delivery, an increase in leukocyte alkaline phosphatase activity from 111.5 just before delivery to 285 mg. 5 days later was accompanied first by a slight rise and then by a slight fall in the white blood cell

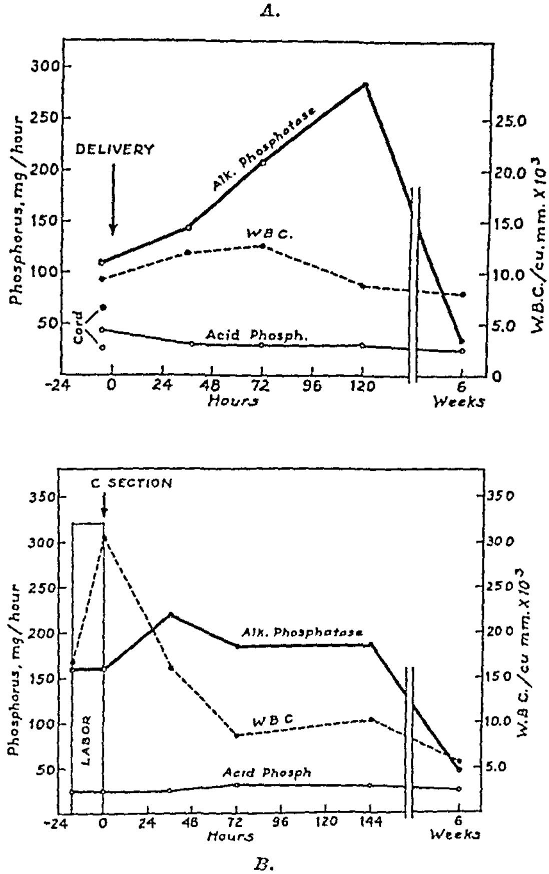

Fig. 2.—*A*, Serial post-partum leukocyte alkaline and acid phosphatase activities and white blood counts from a subject who delivered vaginally as well as cord blood and maternal leukocyte phosphatase activities at delivery are shown. While the alkaline phosphatase activity rose considerably during the early puerperium, only a slight rise in the blood leukocyte [illegible] during the period studied. *B*, Data are from a patient who was delivered after 20 hours of labor because of cephalopelvic disproportion. A marked [illegible] end of labor was not accompanied by a comparable increase in leukocyte activity.

count In the case demonstrated in Fig 2, *B*, a cesarean section was performed 24 hours after the onset of labor because of cephalopelvic disproportion During labor within a period of 20 hours the leukocyte count rose from 16,750 to 31,150 per cubic millimeter, but with this rather intense leukocytosis a comparable increase in leukocyte alkaline phosphatase activity was not detected

The leukocyte alkaline phosphatase activity in cord blood was less than the maternal leukocyte activity at delivery, instead it was quite similar to that in maternal leukocytes during the third trimester, averaging 77 mg in the former and 76 5 mg in the latter In only one instance was the cord blood leukocyte enzymatic activity higher than in the maternal blood obtained at the end of labor

In one of the 2 subjects with a fetus dead in utero 8 weeks after fetal death the leukocyte alkaline phosphatase activity was 56 mg while the leukocyte concentration was 7,080 per cubic millimeter, in the other in whom the fetus was dead for more than 4 weeks the alkaline phosphatase activity was 103 5 mg and the leukocyte count was 13,200 per cubic millimeter

DISCUSSION

It is apparent that during normal pregnancy there is a considerable increase in leukocyte alkaline phosphatase activity The increase usually begins during the first trimester of pregnancy before any change in the concentration of circulating leukocytes is noted It reaches a plateau during the second trimester and then rises rather abruptly during labor and the early post partum period During pregnancy and the early puerperium the degree of elevation of leukocyte phosphatase activity and the increase in blood leukocyte level frequently are not of comparable magnitude Six to 7 weeks after delivery the leukocyte alkaline phosphatase activity approaches the normal nonpregnant level

The mechanism by which the increase in alkaline phosphatase activity is produced is unknown The observation of elevated leukocyte alkaline phosphatase activity in 2 subjects with prolonged intrauterine retention of a dead fetus suggests that the increase in enzyme activity is not dependent upon something elaborated by the fetus

Valentine and associates[3] suggest a relationship between adrenal cortical activity and leukocyte alkaline phosphatase activity since the administration of corticotropin gel intramuscularly or of large doses of cortisone or hydrocortisone orally to normal subjects produces an increase in leukocyte alkaline phosphatase activity

There is considerable evidence of increased adrenal function during normal pregnancy The human placenta contains a material which has many of the activities of ACTH as well as the same electrophoretic pattern as ACTH[4, 5] Gemzell[6] and Bayliss and associates[7] have demonstrated a several fold increase in 17 hydroxycorticosteroid concentration in plasma during pregnancy A further increase is noted following labor according to Migeon and co workers[8]

These observations suggest but do not prove that the rise in leukocyte alkaline phosphatase activity during normal pregnancy is related at least in part to an increased production of corticoids during normal pregnancy

SUMMARY

1. Leukocyte alkaline and acid phosphatase activities were measured during pregnancy and the puerperium.

2. Leukocyte acid phosphatase activity did not change during pregnancy and the puerperium.

3. Alkaline phosphatase activity rose early in pregnancy to reach a plateau during the second trimester and then during labor and the early post-partum period it was further elevated.

4. The mechanism which causes this increase is unknown but the possibility that it is related to increased adrenal function during pregnancy is suggested.

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EXPERIMENTAL AMINONUCLEOSIDE NEPHROSIS IN RATS

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INTRODUCTION

THE nephrotic syndrome is characterized by proteinuria, hypoalbuminemia hyperlipemia, and edema In man it is associated with such widely varied pathologic states as glomerulonephritis, amyloidosis, diabetes mellitus, lupus erythematosus, and the administration of the drug, trimethadione[1] Though the fundamental lesion appears to be a renal defect with profound proteinuria, the metabolic pathogenesis of the syndrome is still obscure

Experimental nephrosis has been produced in rats by an immunologic mechanism namely, the injection of antirat kidney serum[2, 3] This disease resembles glomerulonephritis, and its metabolic[4, 5] and histologic characteristics have been amply studied[2, 3] Recently Frenk, Antonowicz, Craig and Metcoff[6] reported that 6 dimethylaminopurine 3 amino d ribose induced nephrosis in rats It seemed pertinent to investigate this drug induced syndrome and to compare it with the experimental immunologic disease

This paper is concerned with a study of aminonucleoside induced nephrosis in rats The course of the disorder, the metabolic and histopathologic changes, as well as studies of adrenal cortical function and response to steroid therapy are described

EXPERIMENTAL

Immature male Sprague Dawley rats weighing 90 to 100 grams were used Twenty six control animals, 7 of which were treated with cortisone, and 27 nephrotic animals, 12 of which also received cortisone, were studied Rats were caged individually in metabolic units After two days of adjustment to the cages and to the Purina rat chow diet, studies were begun Daily subcutaneous injections (0 3 ml per 100 gram rat) of a 0 5 per cent solution of 6 dimethylaminopurine 3 amino d ribose* were given Controls received injections of distilled water Injections were continued throughout the study In the first experiments animals were sacrificed serially from the sixth to the fourteenth day of injection, while in later studies, animals were sacrificed from the twelfth to the fourteenth day Cortisone acetate (2 5 mg per day) was given subcutaneously to the animals indicated from the onset of proteinuria until sacrifice In one study control animals and steroid treated nephrotic animals were pair fed with the untreated nephrotics the latter being provided with food and water ad libitum Animals were weighed daily, and the total daily urinary protein excretion was determined by the Esbach method[7] At the time of sacrifice, rats were decapitated and blood was collected with heparin as anticoagulant

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*We are indebted to the Lederle Laboratories for kindly supplying the nucleoside for chemical determinations. Total and fractional serum proteins were determined by the Lowry microtechnique[8] using a Howe fractionation method.[9] Serum cholesterol was measured by the method of Saifer and Kammerer[10] and nonprotein nitrogen by the method of Koch and McMeekin.[11] Liver glycogen analyses were made immediately on removal of liver tissue by the microtechnique of Waalas.[12] Pooled samples of both serum and urine were subjected to paper electrophoresis, by use of a barbiturate buffer adjusted to a pH of 8.6.

Studies of adrenal cortical function were carried out. Adrenal glands obtained from control, nephrotic, and cortisone-treated nephrotic animals were dissected free of fat, weighed, bisected, and incubated in a glucose-Ringer-phosphate buffer to which one unit of ACTH had been added. At the end of a two-hour incubation in the Dubnoff shaker in an atmosphere of O_2, at 37° C., the quantitative production of steroids was measured by a spectrophotometric method described by Eisenstein.[13] Qualitative identification of these steroids was made by paper chromatography.[14] For histopathologic studies, hematoxylin-eosin, fat stains and periodic acid-Schiff stains were made of sections of kidney. Routine staining procedures were used for other organs studied.

Fig 1.—Appearance of the nephrotic rat.

RESULTS

A. *Course of the Disease.*—Aminonucleoside injections produced a nephrotic syndrome in all animals with remarkable uniformity. Proteinuria appeared as the earliest manifestation and was followed by accumulation of ascites. In Fig. 1 the characteristic appearance of the nephrotic animal may be seen. After these manifestations had appeared, cessation of injections did not alter the outcome of the disease. None of the animals survived beyond the sixteenth day.

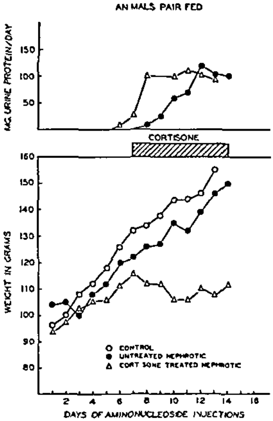

Fig 2 —Effect of aminonucleoside injection on weight curves and proteinuria in pair-fed animals.

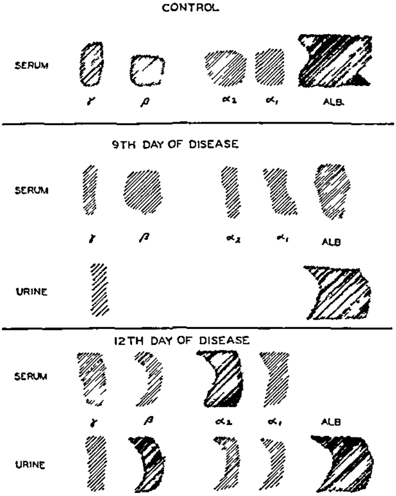

Fig 3 —Diagrammatic representation of paper electrophoretic patterns of pooled urine and serum samples obtained from animals injected with aminonucleoside

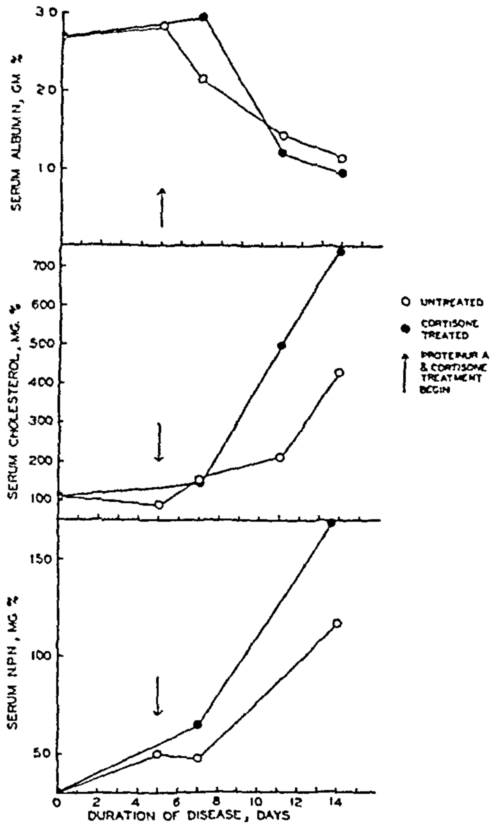

Fig 1.—Progressive changes in serum albumin, cholesterol, and nonprotein nitrogen with injection of aminonucleoside

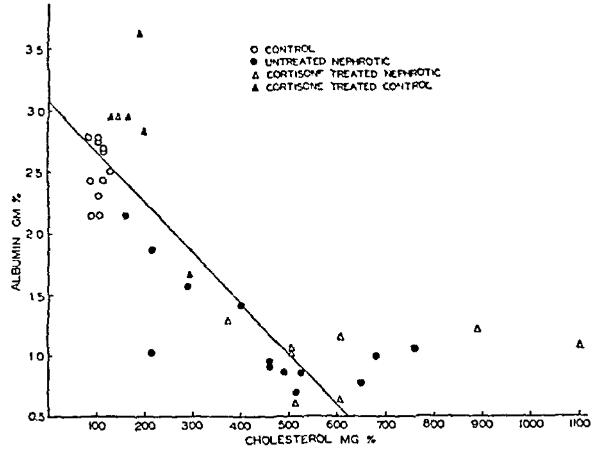

Fig 5.—Relationship between serum cholesterol and albumin levels

Random urine examinations revealed proteinuria, homogeneous and granular casts, and a few white blood cells Neither red blood cells nor glucose were present

Weight curves during the course of the disease are shown for pair fed animals (Fig 2) It may be seen that, despite rapidly accumulating ascites, after the tenth day, the slope of the weight curve of the nephrotic animals did not change markedly, indicating probable tissue protein losses as fluid was retained Administration of cortisone did not favorably affect the course of the disease A striking inhibition of weight gain was noted in steroid treated animals, though ascites was present in every case (Fig 2)

Proteinuria Proteinuria began between the sixth and eighth day of injection, usually becoming massive by the tenth day (Fig 2) No animal excreted less than 60 mg protein per day The average excretion was of the order of 100 mg per day Electrophoretic patterns of the urinary proteins indicated that albumin appeared in the urine first and was followed in later stages of the disease by the excretion of alpha, beta, and gamma globulins Serial electrophoretic patterns obtained from pooled urinary samples may be seen in Fig 3 No significant differences, qualitative or quantitative, were noted in the proteinuria of steroid treated rats

TABLE I

	CONTROLS	CONTROLS + CORTISONE	NEPHROTICS	NEPHROTICS + CORTISONE
Serum Albumin (Gm %)	2 7 + 62 (13)	2 86 + 58 (5)	1 2 + 46 (14)	1 2* ± 69 (9)
Total Serum Protein (Gm %)	4 8 + 50 (16)†	4 89 + 63 (7)	3 24 ± 79 (14)	3 18 + 98 (12)
Cholesterol (mg %)	109 + 26 (17)	162 ± 38 (7)	452 + 175 (15)	561 ± 244 (12)
Nonprotein Nitrogen (mg %)	27 5 ± 17 (19)	26 ± 13 (5)	115 ± 48 (13)	82 ± 91 (9)‡
Liver Glycogen (mg /Gm wet weight)	22 2 ± 16 (19)	43 2 ± 13 (5)	8 5 + 3 2 (9)	15 4 + 13 4 (7)

*Mean + standard deviation
†Number of animals
‡One spuriously high value included

Hypoproteinemia The level of serum albumin fell strikingly in the aminonucleoside injected animals following several days of proteinuria (Fig 4) Table I shows the mean level of serum albumin and total protein in all the animals studied It is noteworthy that cortisone did not alter the protein levels in nephrotics In Fig 3 the qualitative electrophoretic changes in serum proteins may be seen Albumin levels decrease while α_2 globulin increases markedly It is of interest that these patterns closely resemble those in human nephrosis Similar changes occurred in cortisone treated nephrotic rats

Hypercholesterolemia The concentration of serum cholesterol increased significantly as the syndrome progressed (Fig 4) Elevations appeared to correlate with the serum albumin decrease (Fig 5) Table I shows the mean change noted in all the groups studied. A higher level of cholesterol was observed in all cortisone-treated animals as compared with their controls, but this difference did not prove to be statistically significant.

Serum Nonprotein Nitrogen and Liver Glycogen Levels: Progressive rise in nonprotein nitrogen without oliguria was noted in all animals treated with aminonucleoside. The levels attained may be seen in Table I.

Liver glycogen levels were uniformly lower (Table I) in the nephrotic animals and this could not be explained on the basis of deficient food intake. Cortisone significantly increased the level of liver glycogen.

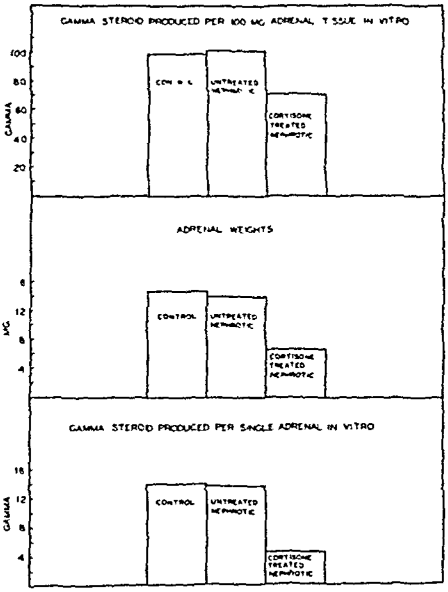

Fig 6.—Studies of adrenal response to incubation in vitro with ACTH.

B. *Studies of Adrenal-Cortical Function in Vitro:* Six controls, six nephrotics, and four cortisone-treated nephrotic animals were studied. The adrenals were incubated in vitro with ACTH and steroid formation was measured. No significant differences in adrenal gland weight or quantitative steroid formation could be demonstrated between control and nephrotic animals (Fig. 6). Cortisone treatment of nephrotic animals resulted in a 30 per cent reduction in steroid formed per 100 mg. of adrenal tissue and a 50 per cent reduction in adrenal weight. It was of some interest that paper chromatography of the steroids produced in vitro showed no abnormal increase in aldosterone in the nephrotic group.

C. *Pathologic Studies.*—Gross: All tissues examined were found to be edematous and pale. Ascites and hydrothorax were noted. Abdominal organs appeared normal except for the kidneys. The latter were enlarged and were very pale. The capsule stripped with ease and the *surfaces of the kidneys* were smooth.

Microscopic: Examination of the liver, adrenal, and pancreas revealed no abnormalities. Kidney sections may be seen in Fig. 7. Nonspecific changes in the glomeruli consisting of a frayed appearance of the basement membrane and general fullness of the glomerular tuft are seen in Fig. 7, *B*. In some cases Bowman's capsule appeared distended. Casts were seen in the tubules, and in sections from those animals in the later stages of the disease (Fig. 7, *D*) much fat staining material could be seen in tubular epithelium, endothelium, and blood vessels. It was not possible to describe basement membrane thickening in the PAS stains (Fig. 7, *C*).*

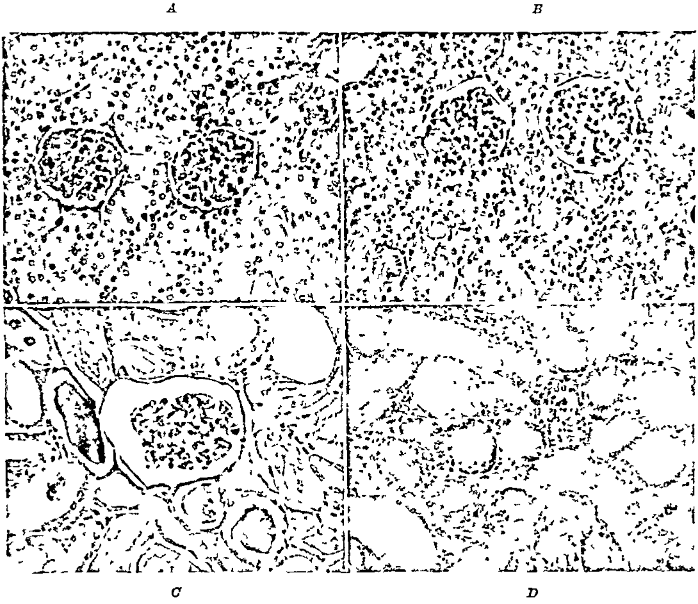

Fig 7.—Histopathologic section of rat kidney *A*, Control, H and E stain, *B*, nephrotic H and E stain, *C*, nephrotic, PAS stain, *D*, nephrotic fat stain

DISCUSSION

The experiments described indicate that 6 dimethylaminopurine-3 amino-d-ribose is a potent nephrotogenic drug in rats A classical nephrotic syndrome was produced over a period of 14 days The sequence of events appeared to be massive protein loss via the kidney, starting on the sixth day, with secondary hypoproteinemia and edema. The hypercholesterolemia developed only when the serum proteins were significantly depressed. The development of the elevated serum nonprotein nitrogen was rather surprising in view of the minimal anatomical changes in the kidney. One is impressed that this drug-nephrosis closely resembles the antikidney serum nephrosis. The changes in the serum and urinary proteins, the hyperlipemia, and the depressed levels of liver glycogen are strikingly similar, despite the different techniques of induction. However, certain differences between these two varieties of nephrosis were observed. Rats made nephrotic with antirat kidney serum have hematuria and usually a deposition of fibrinoid material in and around the basement membrane of the glomerulus, in contrast to the drug-treated group. Perhaps the most striking difference is seen in the response to cortisone. In this study, steroid-treated nephrotic rats were adversely affected by doses of 2.5 mg. per 100 grams body weight. This was particularly notable in their weight curves. As the levels of serum protein, proteinuria, and accumulation of ascites were similar, it is assumed that the decrease in weight gain seen in the cortisone-treated group reflected a decrease in tissue protein. In the studies reported[2, 3] rats made nephrotic with antikidney serum responded favorably to the same quantity of cortisone as we used in our experiments. There occurred a decrease in proteinuria, edema, and in the basement membrane changes.

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*We are indebted to Dr W Stanley Hartroft for interpretation of these sections.

No evidence of adrenal hyperfunction in the drug-nephrosis was observed. The glands were of normal size and hypersecretion of aldosterone could not be demonstrated. This certainly does not rule out endogenous hyperaldosteronism, since these experiments were performed in vitro with exogenous ACTH added. However, Eisentein[15] demonstrated an increase in aldosterone secretion by salt-depleted rat adrenals. These data coupled with the finding of adrenal gland enlargement in the antikidney serum group,[3] as well as the difference noted with respect to cortisone response, suggest that a fundamental difference may exist in adrenal function in these two syndromes. It is not known whether such differences may be found in the variety of human nephroses. There are certainly cases of human nephrosis which do not respond to steroids. The question, however, of dosage of steroid must be raised both in the human and the animal experimental group. It is possible that larger doses might result in a favorable response.

The mechanism by which the aminonucleoside syndrome is induced presents an intriguing problem. The probability that the nucleoside produces profound enzymatic aberrations seems likely in view of the fact that there is so little evidence of morphologic changes in the kidneys and other tissues. It seems reasonable, therefore, to consider the chemical nature of this drug and to examine its potential role in metabolism.

Puromycin, an experimental antibiotic obtained from *Actinomyces alboniger*, is the parent compound of the aminonucleoside. In Fig. 8, the chemical structures of both of these compounds as well as of adenosine, a physiologically important purine, are shown. It is apparent that they both resemble adenosine and differ from each other only in that the nucleoside has a free amino group on the ribose moiety, while Puromycin has the amino group tied up in amide linkage. Both Puromycin and the nucleoside have been reported to be effective against experimental parasitic infections (trypanasomiasis, amebiasis)[16, 17] and also against experimental tumors in mice.[18] Puromycin has been used in several cases of far advanced human tumor.[19] Studies of the pharmacology and toxicity of Puromycin in rats indicate that the drug has a low level of nephrotoxicity.[20] Ascites and renal casts have been described only with the *intraperitoneal administration of doses of 10 mg per 100 gram rat for ten* days or longer. In the experiments with the nucleoside, doses of less than 1.5 mg. per 100 gram rat produced a striking nephrotic syndrome. It seems likely that the structural difference, namely the free amino group in the nucleoside, is in some way related to the potency of the nucleoside as a nephrotogenic agent. The similarity of both drugs to adenosine suggests that they may function as competitive analogues of adenosine. At the present time, studies of the mechanism of action of the nucleoside are under way in our laboratory.

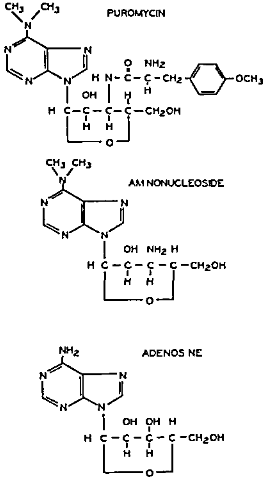

Fig. 8.—Chemical structure of adenosine, aminonucleoside, and Puromycin.

SUMMARY

Experimental nephrosis has been produced in rats by the administration of 6-dimethylaminopurine-3-amino-d-ribose. The course of the disease, certain of the metabolic changes, and the histopathology have been described. No evidence of response to steroid therapy was obtained. The possible mechanism of action of the drug has been discussed.

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DIFFERENTIAL DIAGNOSIS OF GASTRIC ULCER AND CANCER BY A STUDY OF MAXIMALLY STIMULATED GASTRIC SECRETION

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THE differential diagnosis of benign and malignant lesions of the stomach remains a difficult problem despite the number of diagnostic aids that are available In any of these, the overlap between values obtained in these two conditions is sufficiently large to leave room for considerable doubt[3] This applies particularly to the variety of gastric secretory function studies used—direct gastric analysis for a study of acid content, employing test meals, alcohol, or a single dose of histamine, and indirect gastric "analyses" using dye or quinonium exchange resins, and the plasma and urinary pepsinogen estimations Radiology and gastroscopy and gastric cytology are superior to gastric function tests, but still fail to clearly distinguish ulcer from cancer in a moderate number of cases

This report describes the results of an attempt to distinguish gastric ulcers from gastric cancers by employing a maximum stimulus of gastric secretion in the form of an intravenous infusion of histamine This test has permitted the differential diagnosis between ulcer and cancer with a high degree of certainty

METHODS

The study comprised 15 patients with cancer of the stomach, proved histologically in 13 and by gastroscopy and radiology in 2, one of whom had in addition a cancerous node in the neck proved histologically, and 16 patients with benign gastric ulcer, 3 proved histologically and the remainder followed radiologically or gastroscopically to complete healing All had been hospitalized The age of the cancer group was 38 to 78 years (mean 61 years), and in the ulcer group 25 to 70 years (mean 51 years)

The gastric analyses were performed in the fasting state Levine tubes were passed orally or nasally according to the patient's preference and in most instances were positioned fluoroscopically at the greater curve approximately opposite the angulus Continuous suction was employed supplemented at 15 minute intervals with syringe suction to complete consecutive 15 minute samples After four 15 minute samples in the unstimulated (basal) state an infusion of histamine (5 5 mg histamine diphosphate [≡ 2 mg histamine base] in 1,000 ml physiologic saline to which had been added 20 mg Benadryl to prevent side effects) was started and given at the rate of 65 to 100 drops per minute for four to eight 15 minute periods additionally The patients flushed within a few minutes and the rate of infusion, which was not critical, was adjusted to keep the patient flushed The Benadryl kept the patients a little drowsy In two instances in about 120 such studies, neither of

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them in this series, minor side effects were encountered—one subject had a severe headache and one some bronchospasm. Both were relieved within 5 minutes by the additional intravenous injection of 10 mg. Benadryl. In no instance was it necessary to discontinue the test prematurely. An advantage of this form of histamine administration is that the effects of histamine (flushing and gastric stimulation) cease within 3 to 10 minutes after the infusion is stopped.

Maximal gastric secretion was apparently maintained as long as the patient was flushed (usually 60 to 80 drops per minute) and further increase to 120 drops per minute in 6 experiments caused little or no change in volume or composition of gastric juice. This steady state of maximal gastric secretion can be maintained up to 6 hours without decline of output of acid or pepsin.[1] Reproducibility: One of the cancer patients was studied on five separate occasions at two-week intervals, and though the basal secretion varied considerably, secretion under histamine stimulation produced the same maximal plateau within 2 to 3 meq. of $[H^+]$ and $[Cl^-]$ 5 ml. of volume per 15 minutes, and 100 P.U. per milliliter, on each occasion. Similar observations were made in five normal subjects, to be reported elsewhere.

Free hydrogen ion concentration ($[H^+]$) was measured by electrometric titration (end-point pH 3.3) against 0.1 N NaOH; total chloride ion concentration ($[Cl^-]$) was measured potentiometrically employing 50 per cent $AgNO_3$ delivered from a microburette into a 2 ml. sample of gastric juice in approximately 10 ml. of ±1.0 N HNO_3. Pepsin concentration (P.U./ml.) was measured by the hemoglobin method previously described.[1] Measurements of pH were made with the sealed glass electrode.

RESULTS

The results could not be related to the age of the patient or the duration or extent of the disease.

The volumes of gastric juice per 15 minutes and the concentrations of free acid, total chloride, and pepsin are shown in Figs. 1 to 4 and in Table I. All the samples obtained in the basal state and all the samples in the stimulated state, with the exception of the first sample after starting histamine, in all 31 patients, have been included in the graphs.

TABLE I. MEAN VALUES FOR VOLUME AND COMPOSITION OF GASTRIC JUICE IN GASTRIC ULCER AND CANCER

TYPE OF SAMPLE	DISEASE	NO. OF SAMPLES	VOLUME (ML./15 MIN.)	P.U./ML.	FREE H (MEQ./L.)	TOTAL H (MEQ./L.)	TOTAL Cl^- (MEQ./L.)
Basal	Cancer	59	17.3	387	2.2	7.4	88.6
	Gastric ulcer	51	20.0	816	21.3	29.3	111.0
	% Overlap*	--	97.0	88.0	79.0	96.0	91.0
Stimulated	Cancer	118	23.3	600	27.0	36.0	110.8
	Gastric ulcer	95	36.8	1,140	102.2	109.0	151.0
	% Overlap	--	87.0	65.0	21.0	18.0	13.0

*The percentage of the total number of samples which fall in the area of overlap between ulcer and cancer indicate the chance that any one sample could have come from either group. (See also Figs. 1 to 5.)

Volume of Gastric Juice (Fig. 1).—While the 15-minute volumes of gastric secretion in the basal state were almost identical in the two diseases, the mean volume during histamine stimulation in the cancer group was only 63 per cent of that in the benign ulcer group (Table I). The overlap of values, however, was such as to make the difference in volumes meaningless for differential diagnosis.

The Concentration of Pepsin in Gastric Juice (Fig. 2).—Though the mean concentration of pepsin in the basal state was 2½ times and in the stimulated state almost twice as large in the benign as in the malignant lesions (Table I), the overlap (Fig. 2) of 88 per cent and 65 per cent, respectively, robs this measurement of any value for differential diagnosis.

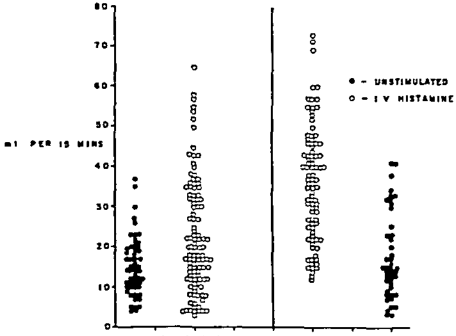

Fig 1—Volume of gastric juice secreted in the basal state and during histamine infusion. Each point represents one 15-minute sample

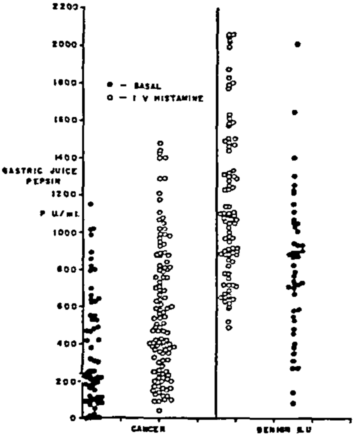

Fig 2—Concentration of pepsin in gastric juice (P U/ml) Each point represents a single 15-minute sample in the respective group

The Concentration of Free H⁺ (Fig. 3).—Differences between the benign and malignant lesions in the concentration of free H^+ were more obvious than differences in volume or pepsin concentration. In the unstimulated state, 52

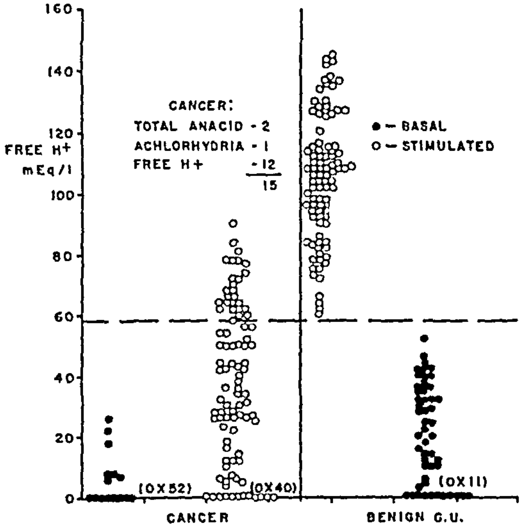

Fig. 3.—The concentration of free acid (<pH 3.3). Each point represents a single sample. In the stimulated groups the first sample after the start of histamine infusion has been eliminated.

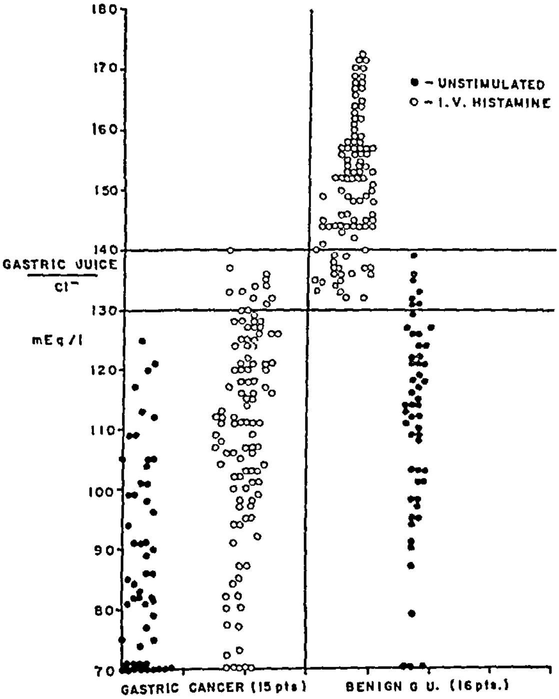

Fig. 4.—Chloride concentration in gastric juice. The normal response to stimulation is an elevation, whereas in the cancer group the chloride concentration corresponds to the range of the normal unstimulated samples.

of 59 samples contained no free acid in the cancer patients, whereas 11 of 51 samples in the ulcer group contained no free acid but the overlap was such that 80 per cent of samples could have come from either group. In the stimulated state, 40 of 118 samples contained no free acid in the cancer group and the maximum concentration reached was 90 meq./L. In the ulcer group the free acid ranged from 60 to 145 meq./L. and the overlap was 21 per cent of all samples, occupying the range of acid from 60 to 90 meq./L., leaving some room for doubt in the differential diagnosis. The maximum free H^+ concentration (Table II) was 10 meq./L. in 8 of the cancer patients, in the other 7 the maximum ranged from 60 to 90 meq./L.

TABLE II. MAXIMUM VALUES IN EACH CASE OF H^+, Cl^-, AND PEPSIN CONCENTRATION UNDER MAXIMAL STIMULATION. THE VALUES IN EACH COLUMN DO NOT NECESSARILY COME FROM THE SAME PATIENTS IN THE ORDER IN WHICH THEY ARE PRESENTED

MAXIMUM H^+/MEQ./L.		MAXIMUM P.U./ML.		MAXIMUM Cl^-/MEQ./L.	
GASTRIC ULCER	CANCER	GASTRIC ULCER	CANCER	GASTRIC ULCER	CANCER
145	90	2,800	1,480	174	140
144	84	2,300	1,420	173	136
139	81	2,300	1,300	163	134
130	78	2,300	1,210	161	133
130	77	1,830	1,150	159	132
126	52	1,630	1,080	157	130
115	60	1,460	1,000	157	126
114	10	1,320	900	156	114
112	6	1,300	760	154	113
110	6	1,280	650	153	112
108	6	1,230	570	149	112
106	4	1,140	570	148	108
104	0	1,090	410	145	106
102	0	970	360	144	100
90	0	930	250	140	82
84				139	

The Concentration of Total Chloride in Gastric Juice (Fig. 4).—The characteristic normal response of the concentration of chloride in gastric juice in man to stimulation is an increase from a level usually below 135 meq./L. in the basal state to a level between 135 and 175 meq./L. in the stimulated state. This characteristic has been observed in gastric ulcers (Fig. 4), in duodenal ulcers, and in normal subjects (unpublished observations). In sharp contrast to this response, the patients with gastric cancer, under maximal histamine stimulation, have levels of gastric juice chloride which correspond almost exactly to the levels of chloride in the normal unstimulated gastric juice (Fig. 4). The overlap between the two groups of all stimulated samples was only 13 per cent of the total.

Differential Diagnosis by Combined Use of [H^+] *and* [Cl^-].—In plotting the concentrations of free H^+ and total Cl^- (Fig. 5) under maximal histamine stimulation, the first 2 samples after starting histamine were excluded so as to be sure of using only unmixed stimulated samples. Of the samples from benign ulcers, all but one fell into the area of the graph defined by the lower limits

135 meq./L. Cl^- and 80 meq./L. H^+. All the samples from the cancer patients fell outside these combined limits. This allowed 100 per cent differentiation between these two conditions in the 31 patients reported here.

Achlorhydria, Anacidity, and Achylia.—If achlorhydria is defined as the absence of free but the production of combined acid (pH 3.5-6.5) and anacidity as the inability to produce any acid (pH 6.5-7.0) with or without stimulation, one patient had achlorhydria (pH 4.6) and two had anacidity, while the rest all produced free acid (pH < 3.3). However, no patient in either group had achylia (the absence of acid *and* pepsin, as in pernicious anemia), since the two patients with anacidity had pepsin levels about 10 times as great as those found in pernicious anemia.

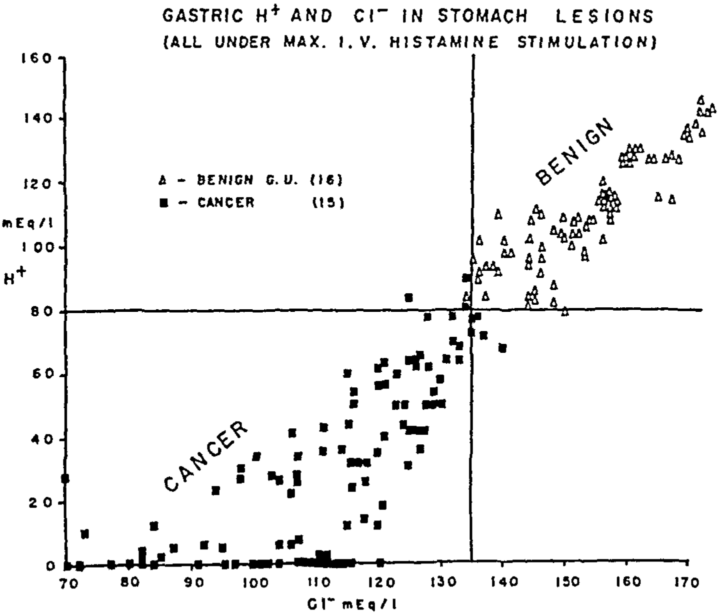

Fig. 5.—Combined plot of H+ and Cl− of all samples of gastric juice in the stimulated state with the first two after starting histamine infusion eliminated. This shows virtually complete separation between cancer and ulcer.

Exceptions to the Rule.—One patient, not included in the above descriptions, who had a bleeding duodenal ulcer of long duration and who also had carcinoma in the stomach, omentum, and liver, provided the one exception in this study. He had a 15-minute volume of gastric secretion in the basal state of 117 ml., free H^+ concentration of 134 meq./L., Cl^- concentration of 157 meq./L., and pepsin concentration of 640 P.U./ml. No further increase could be obtained with histamine stimulation. This hypersecretion in the "basal" state exceeded the values obtained in most duodenal ulcer subjects under maximum stimulation. No explanation can be offered for this phenomenon.

If hyposecretion be defined as the response to histamine which falls in the lower left-hand quadrant in Fig. 5, the same response has been found in one apparently healthy subject, in 2 patients with complete vagotomy, and in a patient with marked malnutrition and signs of pyridoxine deficiency No instance of an inadequate response to stimulation has been observed in any patient with benign peptic ulceration of the esophagus, stomach, or duodenum

Histology—While the appearance of the mucosa varied considerably, no stomach showed either advanced mucosal atrophy or extensive carcinomatous replacement No obvious correlation between structure and function could be found No special studies other than routine hematoxylin and eosin preparations of the specimens were undertaken

DISCUSSION

The composition of gastric juice in the unstimulated state differs considerably from that found in the maximally stimulated stomach Since the techniques of collection of gastric juice are such that the stomach is not entirely drained any transient stimulation will produce a gastric juice of apparently low H^+ Cl , and pepsin, because of unavoidable admixture of basal and stimulated secretion This applies especially to the single subcutaneous injection of histamine The principal objection to the test meal, whatever its composition is that a fixed volume of fluid of unknown chemical composition is added to a varying volume of gastric secretion The resulting mixture, of uncertain volume because of loss through the pylorus, defies any attempt to define the true contribution of the stomach Because of the large empirical experience, however, some standards have been obtained (Osterberg, Comfort) The physiologic knowledge gained from such studies, however, is similar to that which could have been acquired of renal function, if every urine sample were mixed with a bowl of cooked breakfast food before analysis

These objections to the currently employed techniques for gastric secretory function studies are such as to cast doubt on the reportedly high incidence of achlorhydria in normals and in patients with gastric lesions (for a full discussion see the excellent review by Comfort) and led to the search for a technique which would supply some measure of the maximum secretory capacity of the stomach As could have been predicted, such a test would drastically reduce the finding of achlorhydria and eliminate the low values for gastric secretion in normal stomachs This was the finding in normals in patients with duodenal (unpublished) and gastric ulcer, who differed only in the volume of gastric juice but not in its composition The qualitative differences between normal and abnormal mucosae have not been fully evaluated because of the relatively small number of conditions studied and would have to be delineated further for age and sex, for spontaneous variations in normals and for the various disease states which affect the stomach At present in a very limited study, the qualitative difference delineated by the chloride "threshold" of 130 140 meq /L between normal basal and normal stimulated states has sorted out the cancerous stomach and the vagotomized stomach as hyporeactors

Quantitative differences in volume secretion between individuals probably depend on the mass of functioning gastric mucosa, so that it can be expected that children would have less gastric secretion than adults and that the volume would again decline in old age. This information would have to be sought in a larger group of subjects than studied here.

The test of maximal secretory capacity described here has shown no evidence of anything approaching achlorhydria in any of the 16 patients aged 25 to 70 years (mean age, 51 years) and anacidity in only 2 of 16 cancer patients. These were patients with advanced cancer, 10 being inoperable at the time of the study. From this figure of 2 patients with anacidity out of 16, one could say with 95 per cent confidence that between 2 and 40 per cent of all cancer patients would have anacidity at the time of clinically advanced cancer. Assuming the actual percentage found anacid in this study (12½ per cent) it seems unlikely that more had anacidity earlier on, since acid secretion decreases with the advance of cancer (Comfort). It is possible, therefore, to visualize a failure to predict 80 per cent of eventual cancers of the stomach if cancer surveys restrict themselves to the anacid patient. The achlorhydric patient, however, does have an 8 to 10 times greater chance of getting cancer than the patient with free acid by the tests described by Comfort. It remains to be seen whether the qualitative abnormality such as was found in cancer patients in this study precedes or follows the cancer.

The principal use of this test at present, however, is in the differential diagnosis of gastric lesions. It is probable that, as larger groups are studied, more overlap between cancers and ulcers will become evident, but from the preliminary evidence in 31 patients the test appears to offer more than the other conventional tests of gastric function.

SUMMARY

The maximum gastric secretory response to intravenously infused histamine was measured in 31 patients, comprising 15 with cancer and 16 with benign gastric ulcer. While the mean volume of gastric juice and mean pepsin concentrations differed significantly, the overlap of values between the two groups made these two measurements valueless for differential diagnosis. The concentrations of free H^+ showed less overlap and of total Cl^- even less. The combined use of H and Cl concentrations allowed differentiation between ulcer and cancer with a high degree of certainty.

By maximal stimulation of gastric secretion only 2 of 16 patients with cancer had anacidity—an incidence lower than reported found with other types of gastric analysis.

We are grateful for the technical assistance of Mrs. Esther Dullaert and Mrs. Sararuth McCool.

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DIFFERENTIATION OF POLYCYTHEMIA VERA AND SECONDARY POLYCYTHEMIA BY ARTERIAL OXYGEN SATURATION AND PULMONARY FUNCTION TESTS

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THE differentiation of polycythemia vera from secondary polycythemia is sometimes a difficult task. Ratto, Briscoe, Morton, and Comroe[1] have discussed the theoretical reasons that should make this distinction possible by measuring arterial oxygen saturation. Also they point out the practical obstacles. The hypothesis is this: Uncomplicated polycythemia vera should not lead to arterial hypoxemia. Reduced arterial oxygen saturation in a patient with polycythemia should indicate polycythemia secondary to hypoxemia. This conclusion is questionable, however, for the following reasons: (1) Patients with polycythemia vera usually are more than 50 years of age, and healthy persons in this age group may have slight reduction of arterial oxygen saturation.[2] (2) Patients with polycythemia vera may have concomitant lung disease which produces arterial hypoxemia. (3) Most patients with polycythemia vera whose arterial blood has been studied have no evidence of arterial hypoxemia,[1, 3] but there are reports of arterial hypoxemia which is considered to be secondary to uncomplicated polycythmia vera.[4-6] Because of doubt about the interpretation of arterial hypoxemia in patients with polycythemia we decided to undertake the present study. This paper reports studies of arterial blood and pulmonary function in 13 patients with polycythemia vera and 9 patients with secondary polycythemia.

Patients who had a hematocrit of 60 per cent or greater were considered polycythemic. The clinical diagnosis of polycythemia vera was made on the basis of the clinical findings of leukocytosis, high platelet count, and palpable spleen in addition to polycythemia. Conditions capable of producing secondary polycythemia such as cyanotic heart disease, lung disease, high altitude, and respiratory center depression were excluded. Secondary polycythemia was diagnosed when the patient had polycythemia associated with cyanotic heart disease or lung disease in the absence of leukocytosis, high platelet count, and palpable spleen.

METHODS

The 22 patients were seen at the University Hospitals or Veterans Hospital in Iowa City. Each patient had a complete history, physical examination, and the indicated laboratory tests. The red blood cell, white blood cell, and platelet counts, hemoglobin, and

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TABLE I. PHYSICAL CHARACTERISTICS OF AND HEMATOLOGIC FINDINGS IN PATIENTS WITH POLYCYTHEMIA VERA

PATIENTS	AGE (YEARS)	SEX	HEIGHT (INCHES)	WEIGHT (POUNDS)	B.S.A.* M.²	RED BLOOD COUNT† (MILLION/C. MM.)	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT (%)	WHITE BLOOD COUNT (CELLS/C. MM.)	PLATELET COUNT (THOUSAND/ C. MM.)
P. B.	50	M	66	138	1.70	7.75 (5.20-10.10)	24.0 (15.1-24.0)	76 (50-81)	9,800 (9,800-16,400)	212 (98-226)
B. J.	53	F	66	136	1.68	7.96 (6.00-7.96)	22.5 (15.6-22.5)	68 (54-68)	9,850 (8,000-13,700)	316 (102-516)
G. A.	62	F	64	112	1.52	6.47 (4.68-7.22)	19.9 (13.8-19.9)	69 (47-70)	22,300 (22,300-32,250)	336 (159-453)
C. Q.	52	M	68	130	1.68	7.87 (4.45-9.53)	19.0 (13.6-19.0)	65 (49.66)	12,850 (11,000-33,000)	392 (114-710)
W. U.	63	M	72	174	1.99	6.17 (4.23-6.17)	20.4 (11.0-20.9)	70 (40-70)	10,300 (9,200-17,200)	294 (230-294)
L. W.	62	M	68	156	1.83	8.96	19.5	70 (53-70)	36,750 (36,750-58,000)	723 (723-892)
A. M.	51	F	64	143	1.68	7.00 (6.35-7.00)	16.0 (15.9-16.5)	77 (58-77)	17,900 (8,000-17,900)	830
I. M.	44	F	60	124	1.51	6.00 (5.46-8.29)	19.2 (14.0-23.4)	69 (69-71)	9,850 (5,900-13,950)	120 (88-496)
H. H.	64	M	66	130	1.64	8.65 (6.59-8.65)	18.0 (13.1-19.8)	66 (48-66)	28,700 (11,850-28,700)	314 (184-314)
L. M.	42	M	68	174	1.91	5.95 (5.50-6.88)	18.0 (16.7-20.0)	61 (53-67)	13,800 (9,400-16,100)	260 (112-344)
C. V.	69	M	64	165	1.79		15.2 (15.2-16.0)	71 (70-71)	20,600 (13,750-20,600)	162 (96-162)
M. G.	58	F	68	128	1.68	6.50 (5.13-9.04)	21.0 (15.0-21.0	64 (50-64)	6,650 (6,650-10,100)	208 (208-534)
H. B. H.	41	M	71	141	1.80	7.69 (6.00-8.00)	17.5 (16.8-20.7)	59‡	43,600 (26,800-43,600)	489

*B.S.A. = *Body surface area.*

†Top figure is initial blood count. Figures in parentheses represent high and low values of all counts on this patient.

‡After phlebotomy at another hospital.

hematocrit determinations were done in the clinical laboratory of the hospital Hemoglobin was measured colorimetrically by use of a Klett Summerson photoelectric colorimeter Platelet counts were done by the direct method Normal values for platelet counts in this laboratory are 100,000 to 300,000/ c mm

The vital capacity, inspiratory capacity, and expiratory reserve volume were measured separately by a Benedict Roth spirometer The maximum of 3 trials was recorded The functional residual capacity of the lungs was measured in duplicate by the nitrogen washout method of Darling, Cournand, and Richards[7] Volumes were corrected to body temperature, ambient pressure, and saturated with water vapor, hereafter called BTPS Rate, depth, and minute volume of ventilation were measured while the patient was breathing air, 99 6 per cent oxygen, and 7 5 per cent carbon dioxide in air Expired volumes were collected in a Tissot spirometer simultaneously with the drawing of samples of arterial blood Volumes were corrected to BTPS Uniformity of intrapulmonary distribution of inspired gas was checked by the single breath nitrogen test of Comroe and Fowler[8] and the measurement of the percentage of nitrogen at the end of 7 minutes breathing of oxygen as described by Cournand, Baldwin, Darling, and Richards[9] To measure maximal breathing capacity (MBC), the subject breathed for 15 seconds through a mouth piece, low resistance valve, and wide tubing into a Tissot spirometer The highest value obtained with 3 trials was calculated in liters per minute Maximal inspiratory (or expiratory) flow rate was measured between 200 and 1,200 ml of the inspiration (or expiration) according to the method of Danzig and Comroe[10]

Arterial blood was obtained from the brachial artery while the semirecumbent subject breathed air and again after he had breathed 99 6 per cent oxygen for at least 10 minutes The samples were analyzed for total oxygen content, oxygen capacity, and whole blood carbon dioxide content by the manometric technique of Van Slyke and Neill[11] Blood was rotated with air in a tonometer at room temperature for 20 minutes before measuring oxygen capacity Appropriate corrections were made for physically dissolved oxygen and for differences in total hemoglobin in the content and capacity samples Direct measurement of dissolved oxygen was not made, but an approximate calculation was made on the samples collected during the inhalation of oxygen by subtracting the oxygen capacity from the total oxygen content of such blood Arterial blood pH was measured in a closed Cambridge glass electrode at either 37° C or, if done at room temperature, the pH value was corrected to 37° C, using the factor of Rosenthal[12] Plasma carbon dioxide content and P_{CO_2} were determined from pH, oxygen capacity, and whole blood carbon dioxide content by use of the monogram of Singer and Hastings[13]

RESULTS

A *Patients With Polycythemia Vera*—The initials, physical characteristics, and hematologic findings of the 13 patients with polycythemia vera are shown in Table I Ten of the 13 patients had at least one red blood count above 7 0 million/c mm All patients had at least one white blood count above 10,000/c mm , and 10 of 13 had one platelet count above 300,000/c mm On admission 4 patients (P B , W U , I M , and M G) had essentially normal white blood cell counts and platelet counts In these patients the type of the polycythemia was not evident immediately Additional studies were needed to exclude heart and lung disease and establish the diagnosis of polycythemia vera The spleen was palpable in 9 but was not felt in 4 patients (P B , C Q , L M , and M G)

Results of pulmonary function tests and arterial blood studies are listed in Table II A finding of signal importance is that the arterial blood saturation of patients breathing room air averaged 96 2 per cent, ranging from 93 4 to 100 per cent After breathing 99 6 per cent oxygen for at least 10 minutes the arterial blood saturation in all patients came to 100 per cent, and the

TABLE II. PULMONARY FUNCTION STUDIES AND ARTERIAL

PATIENTS	LUNG VOLUMES: VC (ML.)	LUNG VOLUMES: VC (%*)	LUNG VOLUMES: RV (ML.)	LUNG VOLUMES: RV (%*)	LUNG VOLUMES: TLC (ML.)	LUNG VOLUMES: TLC (%*)	LUNG VOLUMES: RV/TLC × 100	MINUTE VOLUME OF VENT. (L.)	ALVEOLAR GAS DISTRIBUTION: SEVEN-MINUTE WASHOUT (% N_2)†	ALVEOLAR GAS DISTRIBUTION: SINGLE BREATH N_2 TEST (% N_2)‡
P. B.	3,400	89	2,920	120	6,320	96	46	10.0	1.0	
R. J.	3,420	100						7.4		
G. A.	2,650	87	2,830	116	5,480	100	52	7.5	1.5	
C. Q.	2,000**	48	3,420	140	5,420	82	63	15.9	0.5	
	3,020	72	3,420	140	6,440	97	53			
W. U.	4,790	96	3,720	153	8,510	115	44	8.4	2.5	2.0
L. W.	4,020	88	2,870	123	6,890	100	42	8.5	2.0	2.0
A. M.	2,970	88	2,190	90	5,160	92	42	7.7	4.0	2.0
I. M.	2,545	85	2,425	265	4,970	127	49	9.7	2.5	3.7
H. H.	3,190	78	2,160	89	5,350	84	40	7.9	1.5	
L. M.	4,870	102	2,740	229	7,610	126	36	8.6	2.5	1.5
C. V.	3,180	71	2,550	105	5,730	83	45	9.0	0.5	4.0
M. G.	3,165	94	1,650	68	4,815	88	34	10.3	0.0	0.5
H. B. H.	2,550	57	2,200	196	4,750	85	46		0.5	1.5
Mean		85		141		99	44	9.2	1.6	2.2

*% equals per cent of predicted value based on physical characteristics and age.
†Normal values for seven-minute nitrogen washout are less than 2.5 per cent N_2.
‡Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
§MEFR equals maximal expiratory flow rate.

value for dissolved oxygen in arterial blood was normal in all but one patient. These patients had normal P_{CO_2} (31 to 45 mm. Hg, mean 38 mm. Hg) and normal pH (7.35 to 7.41, mean 7.39) of the arterial blood.

Two patients had definite lung disease in addition to polycythemia vera. C. Q. had a pulmonary infarct and H. B. H had a thickened calcified pleura on the left. With the exception of these two patients the vital capacities were normal. The mean vital capacity for all patients including these two was 85 per cent of the predicted normal value. The mean value for residual volume was 141 per cent of the predicted average normal. The mean RV/TLC ratio was 44. The mean total lung capacity was 99 per cent of the predicted normal value. The per cent nitrogen after 7 minutes oxygen breathing was normal in 11 of 12 patients. In 5 of 8 patients the single breath nitrogen test was slightly abnormal, but 4 of these patients were over 50 years of age. Maximal breathing capacity averaged 87 per cent of predicted value, maximal expiratory flow rate 216 L./min. and maximal inspiratory flow rate 189 L./min.

B. *Patients With Secondary Polycythemia.*—The initials, diseases, physical characteristics, and hematologic findings of the 9 patients with secondary polycythemia are shown in Table III. Four patients had at least one red blood count above 7.0 million/c. mm. and all had at least one hematocrit above 60 per cent. Seven had white blood counts persistently below 10,000/c. mm. All 8 patients on whom platelet counts were done had less than 300,000 platelets/c. mm. The spleen was not palpable in any patient.

Results of pulmonary function tests and arterial blood studies are listed in Table IV. In striking contrast to the patients with polycythemia vera not a single patient with secondary polycythemia had a normal arterial oxygen saturation while breathing room air. The values ranged from 62.5 to 90.6 per cent. All patients had definite abnormalities in the other tests depending

BLOOD STUDIES IN PATIENTS WITH POLYCYTHEMIA VERA

MECHANICAL TESTS				ARTERIAL BLOOD STUDIES					
MBC		MEFR§	MIFR‖	O_2 CONTENT REST	O_2 CAPACITY REST	O_2 SATURATION¶ (%)		P_{CO_2} REST	pH REST
(L./MIN.)	(%*)	(L./MIN.)	(L./MIN.)	(VOL. %)	(VOL. %)	REST	100% O_2**	(MM. Hg)	
100	100	298	267	28.17	27.76	100 + 0.43	100 + 2.42	41	7.39
81	100	107	152	25.13	25.14	100	100 + 2.60	45	7.35
68	107	191	210	17.80	18.25	97.5	100 + 1.41	35	7.40
89	91	249	158	16.85	18.04	93.4	100 + 1.71	31	7.38
100	95	334	210	25.01	26.48	94.4	100 + 1.05	39	7.38
77	78	243	211	20.43	21.26	96.1	100 + 2.19	44	7.38
76	97	128	160	23.62	25.04	94.3	100 + 1.30	36	7.37
50	67	137	152	26.17	27.30	95.9	100 + 1.91	33	7.39
85	98	201	191	19.64	20.77	94.6	100 + 0.09	38	7.39
99	81	330	330	23.19	24.27	95.6	100 + 1.89	38	7.41
47	55	187	98	22.45	23.00	97.6	100 + 1.17	35	7.37
75	84	182	146	20.29	21.02	96.5	100 + 1.80	36	7.43
99	84	215	171	19.40	20.43	95.0	100 + 1.34	39	7.40
	87	216	189			96.[illegible]		38	7.39

‖MIFR equals maximal inspiratory flow rate.

¶Values following + sign refer to ml. O_2/100 ml. blood in excess of that required to saturate hemoglobin (i.e. dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

**Results on top line represent findings at the time the patient had chest pain; those on the next line findings 10 days later when chest pain was much improved.

on the basic disease process responsible for hypoxemia. Our patients can be grouped into 3 categories: those with pulmonary emphysema with uneven ratio of blood flow to ventilation, those with congenital heart disease with venous to arterial shunt, and one with pulmonary fibrosis with reduced diffusion across the alveolar capillary membrane.

The 6 patients with pulmonary emphysema had decreased vital capacity, increased residual volume, increase in the RV/TLC ratio, uneven distribution of inspired air, reduced maximal breathing capacity, and reduction in the maximal flow rates. The maximal expiratory flow rate was less than 100 L./min. in 5 of the 6 patients. Four of the 6 patients with emphysema had inadequate carbon dioxide elimination as well as pulmonary insufficiency for oxygenation. After the patients with emphysema breathed 100 per cent oxygen, the arterial blood became 100 per cent saturated and had normal values of 0.70 to 3.00 vols. per cent dissolved in the plasma.

The 2 patients with congenital heart disease with right to left shunt of blood had normal lung volumes, even distribution of inspired air, and normal maximal breathing capacity and maximal flow rates. They had hypoxemia on room air (81.2 and 79.0 per cent), normal P_{CO_2}, but failed to achieve 100 per cent saturation when breathing 100 per cent oxygen. The arterial oxygen saturation was 99.8 per cent in one and 93.1 per cent in the other. Since arterial saturation in these patients was not 100 per cent in addition to 2.00 vols. per cent dissolved in the plasma, some venous blood must have by-passed the lung or gone through the lung without contacting ventilated alveoli. This study could not, by itself, distinguish between an intracardiac shunt and an intrapulmonary shunt. Cardiac catheterization in Patient C. K. demonstrated a patent ductus arteriosus with severe pulmonary hypertension and

TABLE III. PHYSICAL CHARACTERISTICS OF AND HEMATOLOGIC FINDINGS IN PATIENTS WITH SECONDARY POLYCYTHEMIA

INITIALS OF PATIENTS	CLINICAL DIAGNOSIS	SEX	AGE (YEARS)	HEIGHT (INCHES)	WEIGHT (POUNDS)	B.S.A.* M²	RED BLOOD COUNT† (MILLION/C. MM.)	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT %	WHITE BLOOD COUNT (CELLS/C. MM.)	PLATELET COUNT (THOUSAND/ C. MM.)
H. D.	Pulmonary emphysema	M	47	71	171	1.95	6.17	17.1 (15.5-18.4)	58 (49-62)	10,400 (6,400-14,500)	
F. S.	Pulmonary emphysema	M	49	68	143	1.75	7.47 (5.46-7.47)	20.0 (17.0-21.0)	73 (56-73)	8,750 (5,450- 8,750)	80 (80-266)
B. S.	Pulmonary emphysema	M	53	73	268	2.42	7.07	21.0 (16.8-21.0)	70 (60-70)	7,600 (7,600- 9,850)	48
J. S.	Pulmonary emphysema	M	54	72	247	2.31	6.03 (5.48-6.19)	17.8 (16.4-18.1)	65 (52-65)	9,750 (7,200-12,250)	180 (138-180)
H. B.	Pulmonary emphysema	M	59	65	150	1.70	6.91	20.4 (17.3-20.4)	71 (63-71)	6,050 (6,050- 8,650)	120
J. A.	Pulmonary emphysema	M	51	67	204	2.05	6.40 (6.06-6.87)	20.1 (18.0-20.1)	59 (59-60)	7,250 (7,200- 7,250	66 (40- 66)
L. L.	Tetralogy of Fallot	M	35	64	111	1.51	7.54	21.0	65	7,550	208
C. K.	Patent ductus arteriosus	M	49	65	132	1.64	8.76	22.5	70	7,600	134
R. M.	Pulmonary fibrosis	F	52	61	120	1.51	5.98 (5.40-6.80)	17.4 (16.6-22.7)	60 (58-66)	5,400 (5,400- 7,900)	184 (122-184)

*B.S.A. = Body surface area.

†Top figure is initial blood count. Figures in parentheses represent high and low valuesof all counts on this patient.

bidirectional flow through the ductus. Pulmonic stenosis, ventricular septal defect, right ventricular hypertension, and overriding aorta were demonstrated in Patient L. L. by cardiac catheterization and angiocardiograms.

One patient had pulmonary fibrosis. She had hypoxemia completely corrected by breathing 100 per cent oxygen, normal P_{CO_2}, normal vital capacity, even distribution of inspired air, and normal mechanical tests. We were not equipped to measure diffusion directly at the time this patient was studied. Chest x-rays and lung biopsy confirmed the diagnosis of pulmonary fibrosis.

DISCUSSION

We would like to emphasize the fact that our patients with polycythemia vera had arterial blood saturation of 93.4 to 100 per cent. Comroe found that normal arterial oxygen saturation in young adults is 97.5 per cent, S.D. ±2.1. This value compares with 96.5 per cent, S.D. ±1.68, for healthy subjects 50 years and older.[7] The arterial oxygen saturation in all of the patients with polycythemia vera was 93.2 per cent or greater, the difference between this and 97.4 per cent is less than 2 standard deviations. All of the patients with secondary polycythemia were below 93.2 per cent.

In 5 of 8 patients with polycythemia vera the single breath nitrogen test was slightly abnormal but 4 of these patients were over 50 years of age. Griefenstein and associates[9] found values for this test up to 3.0 per cent in healthy persons over 50. Using this value for the upper limit of normal would allow 3 of these patients a normal value for this test.

There is disagreement about whether patients with polycythemia vera have normal or reduced arterial oxygen saturation. Our findings support those of Ratto and co-workers[1] and show that patients with uncomplicated polycythemia vera have normal arterial oxygen saturation. In all the patients with polycythemia and hypoxemia physiologic studies demonstrated an abnormal cardiac or pulmonary mechanism which explained the hypoxemia.

Newman and associates[6] reported pulmonary function studies in 5 patients with presumed polycythemia vera. Two of their patients had arterial saturation of 69 per cent and 65 per cent respectively. Both patients were obese and lethargic. Both had normal leukocyte and platelet counts. Limited pulmonary function studies did not explain the hypoxemia, and a diagnosis of polycythemia vera was made by exclusion. These patients may have had polycythemia vera, but this alone does not explain the hypoxemia. The hypoxemia may have been on the basis of respiratory center depression, as in the case reported by Ratto and co-workers[1], or the patients may have had significant lung disease. The syndrome of hypoxemia, polycythemia, extreme obesity, and hypoventilation has received much attention recently.[1, 14-17] The cause of this Pickwickian syndrome is unknown. Perhaps Newman's two patients were examples of this syndrome.

Cassels and Morse[5] reported oxygen saturation, P_{CO_2}, and pH of arterial blood in 17 patients with "polycythemia vera" and in 9 normal young adults. They stated that "the 17 patients had polycythemia of unknown origin without demonstrable cardiac or pulmonary disease, with one exception (a patient with scoliosis)." Seven of their 17 patients had arterial oxygen saturation

TABLE IV. PULMONARY FUNCTION STUDIES AND ARTERIAL BLOOD

INITIALS OF PATIENTS	LUNG VOLUMES							MINUTE VOLUME OF VENT. (L.)	ALVEOLAR GAS DISTRIBUTION	
	VC		RV		TLC		RV/TLC ×100		SEVEN-MINUTE WASHOUT† (% N_2)	SINGLE BREATH N_2TEST‡ (% N_2)
	(ML.)	(%*)	(ML.)	(%*)	(ML.)	(%*)				
H. D.	2,460	75	3,850	252	6,310	105	63	18.0	3.0	
F. S.	2,350	55						7.1		6.5
B. S.	3,180	50	2,820	116	6,000	77	43	5.1	7.5	3.0
J. S.	2,850	52	3,790	156	6,640	82	70	9.6	9.4	
H. B.	2,230	53	2,430	100	4,660	71	51	6.9	16.0	9.0
J. A.	2,100	41	2,600	107	4,700	62	55	4.6	3.0	2.2
L. L.	2,600	69	1,850	160	4,450	91	41	11.3	1.0	2.3
C. K.	2,875	70	2,900	119	5,775	88	50	7.5	1.0	
R. M.	2,330	85						11.0	1.0	1.0

*% equals per cent of predicted value based on physical characteristics and age.

†Normal values for seven-minute nitrogen washout are less than 2.5 per cent N_2.

‡Normal values for single breath nitrogen test are less than 1.5 per cent N_2.

§MEFR equals maximal expiratory flow rate.

‖MIFR equals maximal inspiratory flow rate.

¶Values following + sign refer to ml. O_2/100 ml. blood in excess of that required to saturate hemoglobin (i.e., dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

below 93.2 per cent. Insufficient data are reported to exclude lung disease in these patients, and even if lung disease could not be identified by clinical or x-ray examination, this does not exclude it as a possible explanation for the hypoxemia.

Harrop and Heath[4] found the diffusing capacity of the lungs reduced in patients with polycythemia vera. We did not measure diffusion in our patients, but Ratto and associates[1] found normal values in 4 of their patients.

Until a basic physiologic disturbance is demonstrated in the red blood cells, the lungs, the respiratory center, or other tissue in patients with polycythemia vera, the best evidence is that arterial oxygen saturation is normal in patients with uncomplicated polycythemia vera. Arterial desaturation is evidence that polycythemia vera is complicated by or exists with another disease.

SUMMARY

We have studied the hematologic, arterial blood, and pulmonary function findings in 13 patients with polycythemia vera and 9 patients with secondary polycythemia. In those with secondary polycythemia hypoxemia was due to emphysema in 6, congenital heart disease in 2, and pulmonary fibrosis in one.

Patients with polycythemia vera usually have high white blood cell counts, high platelet counts, and palpable spleens; but this is not always so. Arterial oxygen saturation with the patient breathing room air was normal in every patient with polycythemia vera.

Patients with secondary polycythemia had elevated red blood cell counts, hemoglobins, and hematocrits in the absence of leukocytosis, increased platelets or splenomegaly. All had arterial hypoxemia. Although it was not immediately apparent in some of these patients that heart disease or lung disease was present, careful physiologic evaluation disclosed the mechanism producing hypoxemia.

Until a basic disturbance of *oxygenation* is demonstrated in patients with uncomplicated polycythemia vera, the best evidence is that the arterial oxygen

STUDIES IN PATIENTS WITH SECONDARY POLYCYTHEMIA

MECHANICAL TESTS				ARTERIAL BLOOD STUDIES					
MBC		MEFR§	MIFI‖	O_2 CONTENT REST	O CAPACITY REST	O SATURATION (%)		Pco REST	pH REST
(L/MIN)	(%*)	(L/MIN)	L/MIN)	(VOL %)	(VOL %)	REST	100% O_2¶	(MM Hg)	
62	54	73	204	21 39	24 06	89 0	100 + 1 93		
33	33	26	54	22 04	27 45	80 3	100 + 1 82	65	7 34
68	50	44	244	19 62	23 85	82 3	100 + 3 00	58	7 32
83	62	100	147	19 87	21 94	90 6	100 + 0 94	45	7 38
19	20	29	143	14 89	23 84	62 5	100 + 0 70	80	7 24
49	40	60	133	18 77	21 74	86 3	100 + 0 90	58	7 33
89	88	277	233	20 17	25 54	79 0	93 1	39	7 38
85	86	490	282	20 50	25 24	81 2	99 8	41	7 38
68	100	155	152	18 90	20 79	82 6	100 + 2 09	38	7 33

saturation is normal Arterial desaturation is evidence that polycythemia vera is complicated by or exists with another disease

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THE EFFECT OF MAGNESIUM SULFATE IN LOWERING TETRACYCLINE BLOOD LEVELS

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ONE of the measures employed several years ago to counteract the gastrointestinal side effects of tetracycline drugs was the administration of aluminum hydroxide gel. It was soon discovered, however, that though it was an effective means of preventing nausea, this practice resulted in a lowering of tetracycline blood levels.[1] The possibility that the effect was part of a more general biologic phenomenon of cation antagonism of tetracycline was suggested by the later discovery that several bivalent cations antagonize the antibacterial action of tetracycline drugs in vitro.[2, 3] It seemed appropriate, therefore, to find out whether another cation which antagonizes tetracycline's antibacterial action might also lower the blood level when the cation and the antibiotic were administered by mouth simultaneously. A study was therefore undertaken in which blood levels were measured at various intervals following the simultaneous administration of tetracycline and magnesium sulfate to man and to rhesus monkeys. As a control, tetracycline was given intramuscularly and magnesium sulfate by mouth to 10 monkeys and 2 human patients. It is the purpose of this communication to report the results.

METHODS

Subjects.—Ten human beings and 20 rhesus monkeys* were studied. The humans, 7 men and 3 women, ranging in age from 39 to 60 years, were patients in the medical wards of the Cincinnati General Hospital. None of the subjects was receiving any other antibiotics. The monkeys weighed approximately 5 kilograms and were healthy. None was receiving medication.

Administration of Drug.—Oral doses of 500 to 1,000 mg. of tetracycline HCl (Achromycin, Lederle) were administered as 250 mg. capsules. Epsom salts ($MgSO_4 \cdot 7H_2O$) were given as a 50 per cent solution. The usual dose of 30 ml. (15 Gm. of magnesium sulfate) was employed except in one instance, when 15 ml. was used. In five instances 30 ml. of castor oil, a nonionic cathartic, was given instead of $MgSO_4$. In every case the magnesium sulfate or castor oil was swallowed by the subject within seconds after the tetracycline.

Tetracycline was administered to the monkeys by stomach tube (No. 18 catheter). With the tube in place, 500 or 1,000 mg. of tetracycline suspended in 20 ml. of 0.01 N HCl

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*The monkeys and the facilities of the Institute for Medical Research, Christ Hospital, Cincinnati, were loaned to us for this part of the investigation through the generosity of Dr. L. H. Schmidt, Director.

was introduced by syringe. The tube and syringe were then flushed with about 25 ml. of water. If Epsom salts were to be administered, 30 ml. of a 50 per cent solution was introduced immediately preceding the tetracycline.

Intramuscular doses of 200 mg. of tetracycline were given in the posterior thigh muscles of the monkeys.

In both the human beings and the monkeys, at least 48 hours elapsed between doses

Collection and Handling of Blood Samples.—Ten milliliters of blood was drawn from the patients by venipuncture immediately before a dose and at 1 and 4 hours after the dose. The blood was placed in sterile centrifuge tubes and allowed to clot at room temperature The samples were centrifuged and the clear serum drawn off and stored at 1° to 3° C. until assayed.

Samples from monkeys were also collected by venipuncture Those receiving tetracycline by stomach tube were sampled just before the dose and at 2 and 3 hours after the dose Those receiving intramuscular tetracycline were bled just prior to the dose and at 1 and 2 hours.

Method of Assay—Each serum sample was assayed twice The first assay was by a twofold serial dilution method, employing *B. cereus* as the test organism

After the approximate antibiotic concentration in the serum had been determined, another aliquot was assayed by means of a measured dilution method in order to determine the level more precisely. Five tenths milliliter of appropriately diluted serum was placed in the first tube, 0.45 ml. in the second, 0 40 ml. in the third, etc. The tenth tube contained 0 05 ml. The volumes were adjusted to 0.50 ml with broth and the tubes inoculated, incubated, and read after 18 hours' incubation at 37° C. A control solution of tetracycline of known strength was run with each assay.

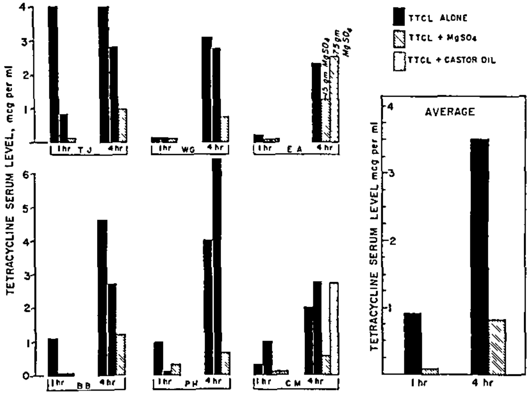

Fig 1—Tetracycline (TTCL) serum levels in human subjects following administration of 500 mg tetracycline HCl by mouth, with and without simultaneous magnesium sulfate.

RESULTS

Lowering of Blood Level by Magnesium Sulfate Administered Simultaneously.—Six human subjects were given 500 mg. of tetracycline alone and, a few days later, the same dose followed immediately by 15 Gm. of Epsom salts. Two separate sets of levels after administration of tetracycline alone were determined in 5 of the patients. In every case except one the blood level when magnesium sulfate was administered was lower at the end of 1 hour than when the tetracycline was given alone. By the end of 4 hours the tetracycline levels were uniformly lower when Epsom salts were given. The magnitude of the lowering was approximately fourfold, i.e., from an average of 0.8 to 0.2 mcg. at 1 hour and from 3.5 to 0.8 mcg. at the end of 4 hours. The experiments are shown in graphic form in Fig. 1. Because of the possibility that the lowering of the blood level might have been due to flushing associated with the catharsis, one patient was given a dose of castor oil along with the tetracycline. The castor oil produced no lowering of blood level. Four patients were given 1,000 mg.

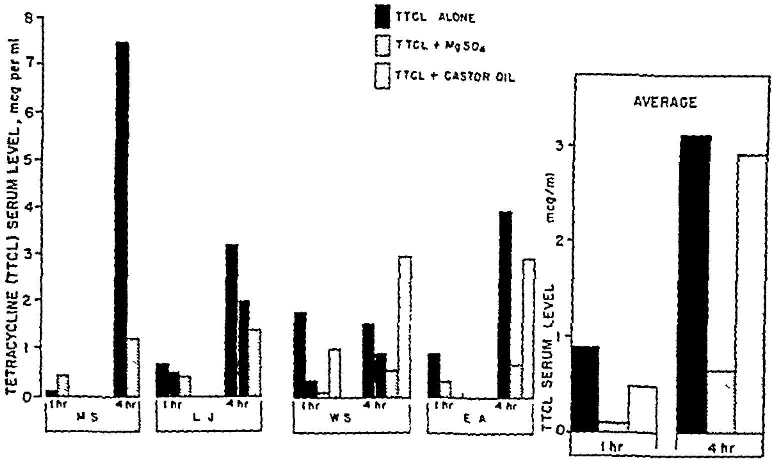

Fig. 2.—Tetracycline (TTCL) serum levels in human subjects following administration of 1,000 mg. tetracycline HCl by mouth, with and without simultaneous magnesium sulfate.

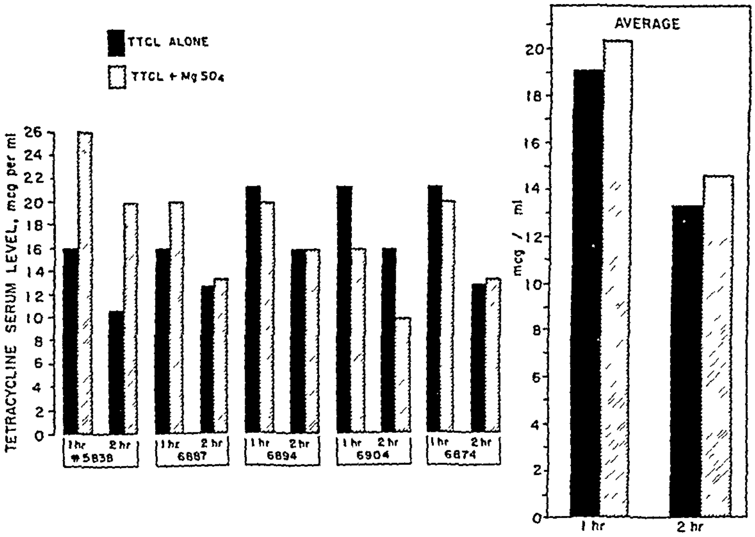

Fig. 3.—Tetracycline (TTCL) serum levels in monkeys following administration of 200 mg. tetracycline HCl intramuscularly, with and without simultaneous magnesium sulfate.

of tetracycline, with and without magnesium sulfate or castor oil The results, as shown in Fig 2, were essentially the same as with the smaller dose A similar lowering of the blood level was found when the study was repeated in rhesus monkeys The lowering effect was found in all of 10 animals studied with a 500 mg dose and all of 5 studied with a 1,000 mg dose

In order to test whether the effect took place in the gastrointestinal tract 5 monkeys were given tetracycline intramuscularly, followed in a few days by tetracycline intramuscularly and magnesium sulfate by stomach tube As shown in Fig 3, *magnesium sulfate by mouth did not lower the blood level* when tetracycline was given intramuscularly A similar study was carried out in 2 stuporous hemiplegic human patients employing a dose of 500 mg of tetracycline intramuscularly Here too, there was no diminution in tetracycline blood level

DISCUSSION

These observations clearly demonstrate that when magnesium sulfate is administered concomitantly with tetracycline given by mouth, the blood level of the antibiotic is suppressed approximately fourfold as compared with the level when no magnesium is given This effect is abolished when tetracycline is given intramuscularly and the magnesium by mouth Whether the effect is dependent upon the failure of absorption of the tetracycline or whether it is explained by the absorption of an inactivated tetracycline is not known Two British chemists, Albert and Rees,[4] have stated that when tetracycline and aluminum hydroxide gel are mixed, a complex is formed which is not absorbed, though they presented no clinical data to substantiate their in vitro chemical experiments Magnesium sulfate itself is very poorly absorbed from the gastrointestinal tract The normal blood level of magnesium for human beings is 1 8 to 2 4 mg per cent, as determined by flame photometry on patients in the Cincinnati General Hospital by Dr James Agna[5] Dr Agna, in 3 separate determinations on patients given 15 Gm of magnesium sulfate by mouth, could find no evidence at the end of 1 and 4 hours of any increase in magnesium blood levels These data do not, however, exclude the possibility that a magnesium tetracycline complex might be absorbed into the blood stream

In our studies the ratio of magnesium to tetracycline in terms of molecular weight was approximately 60 to 1 In one patient where levels were compared employing a smaller dose of magnesium sulfate, giving a magnesium tetracycline ratio of 30 to 1, there was no diminution in tetracycline blood levels Magnesium ion inactivates the antibacterial effect of tetracycline against several species but the ratio of magnesium to tetracycline required to produce this effect is much higher than that observed in our absorption studies Thus, 2,000 atoms of magnesium antagonize the action of 1 molecule of tetracycline when *Pseudomonas aeruginosa* is used as a test organism,[3] 3,000 magnesium to 1 tetracycline when *Staphylococcus aureus* is used,[6] and 50,000 to 1 when *E coli* is used[2] Contrariwise, the chemical avidity of magnesium for tetracycline is said by

Albert[7] to be 1:1 or 1:2, depending upon the pH. It seems evident that magnesium is capable of antagonizing tetracycline, probably by combining with it in some way, but that the ratio of the two substances varies with the conditions under which the reaction takes place.

SUMMARY

When tetracycline and magnesium sulfate were given simultaneously to man or rhesus monkeys, the blood levels of tetracycline were found to be only about 25 per cent of those when the antibiotic was given alone. When tetracycline was given intramuscularly and magnesium sulfate by mouth, no lowering of the blood level occurred. Also, when castor oil was substituted for magnesium sulfate in the oral absorption experiments, there was no lowering of the tetracycline blood level.

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LABORATORY METHODS

A SPECIFIC ENZYMATIC ASSAY FOR THE DIAGNOSIS OF CONGENITAL GALACTOSEMIA

I. THE CONSUMPTION TEST

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CONGENITAL galactosemia is a hereditary disease of childhood characterized by an inability to metabolize galactose. It has been found that the blood and liver of patients with this disorder lack a specific enzyme essential for galactose metabolism. This enzyme, called galactose-1-phosphate uridyl transferase or briefly Gal-1-P uridyl transferase,[1-4] catalyzes the following reaction involving uridinediphospho glucose†

$$\text{I. Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal}$$

The presence or absence of this enzyme in blood can be determined easily; this forms the basis of an enzymatic assay, described in the present report, which can be used to diagnose cases of congenital galactosemia. Such a test eliminates the necessity of subjecting these patients to the deleterious effects of galactose ingestion in a tolerance test.

Human erythrocytes contain a considerable amount of Gal-1-P uridyl transferase. The UDPGal formed in this reaction accumulates without being converted by UDPGal-4-epimerase,[1] formerly called galacto-waldenase,[5] into UDPG. This is due to the fact that hemolysates require the addition of a considerable amount of DPN before the 4-epimerase present becomes active.[6] In the absence of galactose-1-phosphate, UDPG incubated with hemolysates over a period of 30 to 60 minutes is consumed to only an insignificant degree. If galactose-1-phosphate is added in considerable excess over UDPG, most of the UDPG is consumed (i.e., converted into UDPGal). This is partly due to the fact that G-1-P, the other reaction product, is almost completely converted into glucose-6-phosphate by the phosphoglucomutase also present in hemolysates.

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†[illegible] used: UDPG for uridinediphospho glucose; UDPGal for uridinediphospho glucuronic acid; DPN for diphos[illegible]iced diphosphopyridine nucleotide; Gal-1-P for a-galac[illegible] phosphate.

If inorganic pyrophosphate (PP) is added to UDPG incubated with hemolysate there is also a large consumption of UDPG. This is due to the following reaction in which uridine triphosphate (UTP) is formed:

$$\text{II. PP} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UTP}.$$

This enzyme is called UDPG pyrophosphorylase.

Reactions I and II are both measured as the consumption of UDPG in the complete incubation mixture compared with the consumption in hemolysates when UDPG is incubated without the addition of pyrophosphate or galactose-1-phosphate. UDPG is determined in heat-inactivated hemolysates by means of a highly specific dehydrogenase[7] which catalyzes the following reaction:

$$\text{UDPG} + 2\ \text{DPN} \rightarrow \text{UDPGA} + 2\ \text{DPNH}.$$

The increase in optical density at 340 mμ brought about by the reduction of DPN in this manner is a measure of UDPG. It should be emphasized that UDPGal does *not* reduce DPN in this reaction. Since 2 moles of DPN are reduced for each mole of UDPG present, the reduction constitutes a particularly sensitive analytic method. Thus, 0.01 μM of UDPG per ml. gives a change of 0.120 on the logarithmic scale of the Beckman DU ultraviolet spectrophotometer and can be readily determined with an accuracy of about ±10 per cent.[8]

It has been found that hemolysates from humans are rich in enzymes catalyzing reactions I and II.[3, 6] In human congenital galactosemia, however, the enzyme "PGal transferase," catalyzing reaction I, is absent in hemolysate or present in amounts less than 5 per cent of the normal levels.[2, 3] The other enzymes, including the UDPG pyrophosphorylase, catalyzing reaction II, are present within normal range.[3, 6] Hence, if hemolysates are found devoid of the enzyme catalyzing step I, but showing a normal activity with respect to the enzyme catalyzing step II, congenital galactosemia should be strongly suspected. If both enzymes are missing, it is most likely due to improper handling of the blood sample or the hemolysate. The test should then be repeated on another blood sample from the subject.

MATERIALS AND METHODS

Stock Solutions.—

1. Buffers: (a) Tris (tris [hydroxymethyl] aminomethane),* 0.2 M, brought to a pH of 8.1 by means of 1 N HCl. (b) Glycine, 1 M, brought to a pH of 8.7 by means of 5 N NaOH.

2. α-Galactose-1-phosphate, Monobarium salt decomposed with 10 per cent Na_2SO_4; barium sulfate precipitate washed with H_2O and the washwater added to the supernatant, 7.5 μM/ml.,† pH ca. 7.5.

3. Inorganic pyrophosphate, 0.05 M, brought to a pH of ca. 7.5 by means of 1 N HCl.

4. UDPG, disodium,‡ 5 μM/ml., pH ca. 7.5.

5. Cysteine, 210 μM/ml.; neutralized immediately before use to pH 7 or 8 with 1 N NaOH. (Use pH test paper.)

6. DPN, free acid,‡ 50 μM/ml., brought to pH ca. 8.0 by means of 0.1 N NaOH.

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*Obtainable from G. Frederick Smith Chemical Company, 867 McKinley Avenue, Columbus, Ohio.

†Commercial preparation to be available from Sigma Chemical Company, 4648 Easton Avenue, St. Louis, Mo.

‡Obtainable from Sigma Chemical Company, St. Louis, Mo., and Pabst Laboratories, Milwaukee 3, Wis.

7 UDPG dehydrogenase prepared according to Strominger and associates[9] This enzyme preparation should contain 3,000 to 5,000 units per mg protein Keep at 15° C as a solution containing 3 to 5 mg per milliliter Do not lyophilize *

Special Equipment—(a) Microvolumetric pipettes, Linderstrøm Lang Holter † (b) Fused silica cells for Beckman DU spectrophotometer, capacity 1 5 ml dimensions 50 mm. high 5 mm wide and 10 mm. lightpath ‡

Preparation of Blood Sample—Venous blood (in amounts ranging from 2 to 8 ml) is drawn and put into a 12 ml heavy duty Pyrex centrifuge tube containing heparin The blood is mixed with the heparin by inversion of the tube, the opening being covered with "para film " The tube is then cooled by being placed in cracked ice The blood is centrifuged in the cold at 600 × g (full speed, 2,000 r p m , in an International Clinical centrifuge) for 15 minutes and plasma is aspirated off by means of a pipette with a fine tip which is slightly bent The red cells are washed twice with a double volume (i e , two times the volume of the packed red cells) of cold 0 9 per cent sodium chloride and the saline is pipetted off each time in the same way In order to remove the saline as thoroughly as possible, it is advisable to sacrifice the upper layer of erythrocytes The washed red cells are finally hemolyzed by the addition of an equal volume (i e the volume of the packed red cells) of distilled water Pyrex or quartz distilled water is preferable If the hemolysate is transparent, the hemolysis is complete or close to completion Freezing and thawing the hemolysate helps to make the hemolysis complete and does not decrease the activity of the enzyme to be assayed to any detectable degree

Estimation of Red Cell Material as Expressed as Hemoglobin—The amount of cell material present in the hemolysate can be estimated by a hemoglobin determination which is carried out as follows[10] 20 μl of the hemolysate (collected with a micro volumetric pipette) are blown into 5 ml of dilute ammonia (4 ml concentrated ammonium hydroxide to 1 L H_2O), the solution is then mixed, shaken in air to saturate it with oxygen, and read in the Beckman spectrophotometer at 540 mμ § The reading on the logarithmic scale (density scale) times the molecular extinction coefficient of hemoglobin at 540 mμ (14,800)[11] multiplied by the molecular weight (16 700)[11] gives the concentration of hemoglobin in milligrams in the diluted sample Since the sample was diluted 250 fold and since the enzymatic activity is expressed in micromoles substrate converted per hour per gram hemoglobin, the reading at 540 mμ (D_{540}) should be multiplied with the following factors in order to obtain the hemoglobin in grams per milliliter undiluted hemolysate

$$D_{540} \times \frac{1}{14{,}800} \times 16\,700 \times 250 \times \frac{1}{1{,}000} \text{ or } D_{540} \times 0\ 282$$

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*Commercial preparation to be available from Sigma Chemical Company 4648 Easton Avenue St. Louis Mo

†Appropriate for this purpose is a selected constriction pipette obtainable from Mr Pedersen Carlsberg Laboratorium Copenhagen Valby Denmark, or the Arthur H. Thomas Company Philadelphia Pa. (No 2464 H for 50 300 μl and No 464 J for 5 to 40 μl pp 185 186 1950 catalog) See also reference 9

‡Obtainable from the Pyrocell Manufacturing Company New York, N Y

§If utmost accuracy is needed and especially if the hemolysis for one reason or another is not complete the concentration of hemoglobin released from erythrocytes can be estimated in the following way To a 1 ml. aliquot of the hemolysate add 0 1 ml 10 per cent NaCl and mix Spin about 30 minutes at 5 000 to 8 000 r p m (For instance in the high speed attachment head of the International PP 2 refrigerated centrifuge) Take 20 μl of the supernatant and blow over into 5 ml. 0 2 N ammonia and read at 540 mμ for hemoglobin determination The value found here represents the best expression for soluble erythrocyte material This hemoglobin value may be significantly less than that of the sample taken directly from the hemolysate However usually the fraction of nonhemolyzed cells is so small that no special precautions of the kind described here are warranted It should also be noted that whole blood (heparinized) can be used directly since the plasma does not contain any of the enzymes In this case the blood (fresh or stored for 1 to 2 days at 0° to +2° C) is first completely frozen by rotating the tube in a mixture of dry ice and ethanol It is then completely thawed at 37° C once more frozen and once more thawed The hemolyzed blood is then placed in crushed ice and a 20 μl sample is drawn for hemoglobin determination as described for the red blood cell hemolysate (see main text) Hemolyzed whole blood or red blood cells can be stored in a freezing compartment at 10° C for a period of several days without significant loss in the activity of the enzyme If whole blood is being used the deproteinization should be performed with alcohol and chloroform as described previously[3] and not by heating since the latter procedure in this case does not give clear filtrates

Incubation—Centrifuge tubes, 12 ml. heavy-duty Pyrex, are recommended as incubation tubes A mixture for use in all three samples is made with the following composition. 1 ml tris buffer plus 0 1 ml UDPG stock solution Exactly 0 3 ml (a constriction pipette* of 300 μl volume is very suitable for this purpose) of this mixture is placed in each centrifuge tube To sample 1 is added 0 05 ml galactose 1 phosphate. To sample 2 (control sample) is added 0 05 ml distilled water To sample 3 is added 10 μl pyrophosphate stock solution plus 0 25 ml distilled water Finally, the hemolysate is added as follows 0 25 ml to samples 1 and 2 and only 0.05 ml to sample 3 Less hemolysate is added to sample 3 because of (a) the high activity of the pyrophosphorylase and (b) the tendency in this sample for liberation of hemin pigment into the filtrate during deproteinization, making spectrophotometric readings too high The samples are incubated at 37° C for 30 minutes

Deproteinization—At the termination of the incubation period one milliliter of distilled water is rapidly added and the mixture is heated at 100° C for 2 minutes with rapid and constant stirring with a glass rod The sample is then cooled in ice and centrifuged The supernatant is aspirated off for the assay by means of a large constriction pipette It may be necessary to centrifuge the supernatant twice in order to obtain a clear filtrate

An alternative but somewhat more elaborate method, employing chloroform ethanol for deproteinization, and a dual analysis with both UDPG dehydrogenase and UDPGal 4 epimerase, have been described previously [3] The method described here, however, should be fully adequate for routine determination.

Assay—An aliquot of 0 3 ml of the clear supernatant is transferred with a constriction pipette to a Beckman cuvette † The following additions are then made to the sample 0 15 ml. glycine buffer, 0 03 ml neutral cysteine solution, 0 02 ml DPN, 0 5 ml distilled water, and finally 10 μl UDPG dehydrogenase (= 80 units) ‡ The latter is first added to the control, the sample mixed, and the reaction followed in this cuvette § The mixing of the assay mixture with the enzyme is effectively accomplished by placing a piece of parafilm on the top of the silica cuvette and inverting rapidly several times It is particularly important to obtain readings at 340 mμ as early as 20 or 30 seconds after the addition of the enzyme and subsequently at 20 to 30 second intervals during the first 2 minutes after the enzyme addition in order to be able to extrapolate back to the reading at zero time At 2½ minutes the density at 400 mμ is read, and at the termination of the assay another reading at this wave length is made, this is done in order to correct for nonspecific density changes brought about by variations in turbidity or other extraneous factors The readings at 340 mμ are recorded at 6 and 10 minutes and until the density increase signifying reaction has leveled off This usually requires 20 to 30 minutes, some nonspecific increase in density may continue in both samples using certain dehydrogenase preparations, but this may be considered negligible if it is less than 0 005 density scale units per minute Since the initial readings (up to 3 or 4 minutes after the start) and the terminal readings are the crucial ones, it is possible after starting the reaction in the first sample to add UDPG dehydrogenase to the second cuvette 4 to 6 minutes later and likewise 4 to 6 minutes after the start of the second cuvette to add enzyme to the third cuvette The same routine described for the first sample is carried out for the second and third Since a 0 3 ml aliquot corresponds to one fifth of the entire incubate, the values obtained refer to a volume of 50 μl hemolysate

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*Obtainable from Mr Pedersen Carlsberg Laboratorium, Copenhagen Denmark, or the Arthur H Thomas Company Philadelphia Pa

†Obtainable from the Pyrocell Manufacturing Company, New York, N Y

‡One unit is the amount of enzyme which produces a Δ_{340} of 0 001 per minute in 0 5 ml [3] Sufficient dehydrogenase is used to give a Δ_{340} of approximately 0 040 per minute in 1 ml In our preparations this is 0 01 ml but if the enzyme preparation used is less active than this part of the water in the assay can be replaced by additional dehydrogenase Care should be taken when adding the enzyme so that the droplet which is delivered above the surface of the mixture in the cuvette is not blown into foam It is preferable to leave a trace in the pipette in order to avoid this

§The density at zero time at 340 mμ usually amounts to 0 30 to 0 40 However the cuvette containing the filtrate from the sample incubated with inorganic pyrophosphate often shows a density which is somewhat higher (0 50 to 0 70)

Calculation of Gal-1-P Uridyl Transferase Activity.—The difference in ΔD_{340} (increase in optical density at 340 mμ) between the blank and the samples containing galactose-1-phosphate or pyrophosphate constitutes the basis for the calculation of UDPG consumption.* In order to express the value in micromoles substrate converted per hour per Gm. hemoglobin, it is first necessary to transform the density differences at 340 mμ (ΔD_{340}) into micromoles. By the method used 1 micromole per ml. converted would correspond to a ΔD_{340} of 12.0.[8] The micromoles UDPG converted in 1 ml. hemolysate in 1 hour would be $\Delta D_{340} \times 20 \times \frac{1}{12.0} \times 2$ or $\Delta D_{340} \times 3.3$. As mentioned above, the amount of hemoglobin, expressed in grams in 1 ml. hemolysate is derived from the reading of the dilute aliquot at 540 mμ multiplied by 0.282. The enzyme activity expressed in micromoles substrate converted per hour and per Gm. hemoglobin is therefore obtained from the following equation:

$$\frac{\Delta D_{340} \times 3.3}{D_{540} \times 0.282} \text{ or } \frac{\Delta D_{340}}{D_{540}} \times 11.7$$

Calculation of UDPG pyrophosphorylase activity is similar except that the formula is multiplied with a factor of 5 since only one fifth as much hemolysate is used.

TABLE I. ACTIVITY OF PGAL URIDYL TRANSFERASE AND UDPG PYROPHOSPHORYLASE IN RED BLOOD CELL HEMOLYSATES

(Rates expressed as uridyl transfer, micromoles per ml. red blood cells per hr., 37° C.)

PATIENT	PGal TRANSFERASE	UDPG PYROPHOSPHORYLASE
J. W., 6 yr., M., normal	1.80	3.75
T. R., 7 mo., M., normal	1.35*	3.60
A. F., 6 yr., M., normal	2.61	2.46
C. F., 8 yr., M., normal	2.61	
K. F., 7 mo., F., normal	3.00	
B. M., 4 yr., F., normal	>2.25	
B. M., 3 yr., M., normal	2.61	
H. K., 47 yr., M., normal	3.90	
V. McC., 5 mo., M., milk allergy	1.80*	
M. B., 7 mo., M., milk allergy	2.25*	
C. M., 13 mo., M., milk allergy	3.00	4.44
N. J., 6 yr., M., nephritis	2.70	
M. L., 24 yr., F., chorion epithelioma metastic (+ lactation)	2.76	
A. B., 20 yr., M., postnecrotic liver cirrhosis	2.50	

*Reaction run in the reverse direction, i.e., G-1-P + UDPGal $\rightleftharpoons$ Gal-1-P + UDPG.

RESULTS AND DISCUSSION

Transferase Values as Related to Congenital Galactosemia.—The values for the activity of PGal transferase in erythrocytes have been determined in a large number of both male and female subjects of various ages and on various diets; normal subjects as well as subjects afflicted with various diseases have been studied.[3] Expressed per Gm. hemoglobin the average value was found to be of the order of magnitude of 2.5 to 3.5 μM (see Table I). The normal variations are moderate amounting to about ± 30 per cent. It is, however, likely that the average value is actually somewhat higher since the reaction is only linear with time within the first 10 to 20 minutes. In 15 cases of congenital galactosemia we have found that this enzyme is either absent or present in amounts which are so low that it cannot be detected with this method.[3] (Table II.) The method permits the detection of activities as low as 0.1 μM per Gm. hemoglobin and if the incubation time is extended, activities down to 0.05 μM can be detected. In all the cases of congenital galactosemia, regardless of age, sex, or diet, the activity was found to be below 0.05 μM and hence below the detectable level. The other enzymes involved in UDPG metabolism, such as UDPGal-4-epimerase or UDPG pyrophosphorylase, have been shown to be present in normal amounts in congenital galactosemia.[3, 6] Usually the amounts of UDPG pyrophosphorylase are so high that all, or more than 75 per cent, of the UDPG is consumed under the conditions described.

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*It should be noted that the absolute density difference brought about by addition of UDPG dehydrogenase to the control is about 0.400 (Fig. 1), whereas the specimen illustrated in Fig. 2 showed a density difference of 0.300 although the same amount of UDPG was added. The purity of commercial UDPG samples seems to vary somewhat from batch to batch. Moreover, different hemolysates seem to contain different amounts of factors which influence the density increase. These fluctuations, the nature of which are not clear, do not disturb the assay at all. In all the cases of galactosemia, for instance, the difference between the density increases in the two samples is within 10 per cent.

TABLE II. ACTIVITY OF PGAL URIDYL TRANSFERASE AND UDPG PYROPHOSPHORYLASE IN HEMOLYSATES OF RED BLOOD CELLS FROM NORMAL AND GALACTOSEMIC SUBJECTS
(Rates expressed as uridyl transfer, micromoles per Gm. hemoglobin per hr., 37° C.)

PATIENT	PGal TRANSFERASE	UDPG PYROPHOSPHORYLASE
Family O. D.:		
Father, 33 yr., normal	3.36	3.75
Mother, 28 yr., normal	2.19	
Daughter, 6 yr., normal	2.01	3.75
Son, 4 yr., galactosemic	<0.06	7.50
Daughter, 2 yr., 6 mo., galactosemic	<0.06	7.20
Son, 3 mo., galactosemic	<0.06	5.25
Grandfather, 66 yr., milk "intolerance"	2.25	4.50
Family T.:		
Father, 35 yr., normal	1.89	
Mother, 30 yr., normal	1.71	4.20
Son, 6 yr., galactosemic	<0.06	1.75
Family W.:		
Son, 5 yr., normal	2.40	3.48
Daughter, 2 yr., galactosemic	<0.06	3.75
T. B., 24 yr., M., galactosemic	<0.06	5.25
R. N., 2 yr., M., galactosemic	<0.06	4.80
R. T., 3 yr., M., galactosemic	<0.06	6.60

Example.—An example of an analysis of filtrate from a hemolysate of a nongalactosemic hepatitis patient is illustrated in Fig. 1; curves are shown for UDPG incubation with hemolysate with and without further addition of Gal-1-P. DPN is indicator and UDPG dehydrogenase indicator enzyme. In this case close to 90 per cent of the UDPG added was converted into UDPGal.

Fig. 2 shows an analysis of hemolysate from a galactosemic patient. It can be seen that in this case there is no consumption of UDPG even in the presence of a large excess of galactose-1-phosphate.

As indicated by Fig. 1, the consumption of UDPG proceeds rapidly during the incubation and the value found after 30 minutes incubation usually corresponds to a consumption of UDPG which amounts to at least 75 per cent of the amount added. The reaction rate is considerably higher during the first part of the reaction when only a relatively small fraction of UDPG has been consumed or if higher concentrations of UDPG are employed. Curiously enough even a moderate increase in the UDPG concentration brings about a decrease in the rate of transfer; this procedure seems, therefore, undesirable.

Shorter incubation periods and employment of less hemolysate should, however, provide appropriate conditions for studying rates which are strictly linear with time and hence maximal It should be emphasized that the aim of the assay described in this article is not to study detailed kinetics, but to provide a field test for screening individuals with congenital galactosemia from other individuals

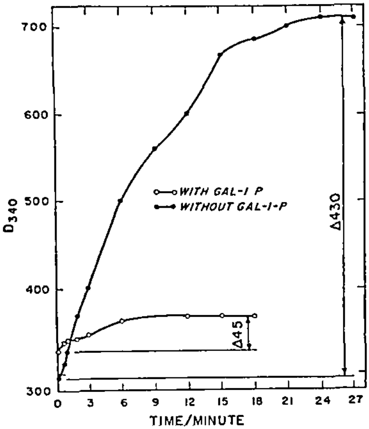

Fig 1 —Analysis of filtrate from a nongalactosemic subject.

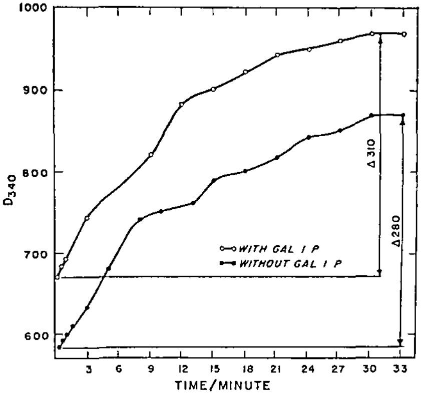

Fig 2 —Analysis of filtrate from a galactosemic subject

These considerations are brought up here since galactose tolerance tests on parents of galactosemic children have revealed that one of the parents usually shows a somewhat lowered tolerance.[12] Thus far we have been unable to detect any strikingly lowered activity of the PGal uridyl transferase with our test. If such a decrease should be present as a trait, it must be of only moderate proportions. It would, therefore, only be detected under conditions which permit a study of maximum rates. Hence, in order to study traits in galactosemia by means of the enzymatic assay, it is mandatory to design the assay in such a way that initial rates can be recorded.

It should be noted that the titer has not been found to be altered in patients suffering from milk allergy or from various kinds of hepatitis, nor was it lowered in two cases of pregnancy observed shortly before term. The latter two conditions have been found to be associated with abnormal galactose tolerance curves.[13, 14] It may be inadvisable to assay blood after injection of large amounts of nicotinamide or nicotinic acid. It is known that nicotinamide and especially nicotinic acid plus glutamine, bring about an increase in the DPN content of erythrocytes,[15] which in turn could cause undesirable side reactions.

The assay can be run quite satisfactorily on cord blood of the newborn[4] and this would seem to be the most feasible procedure for diagnosis of galactosemia in cases where familial history would indicate that it might be expected in the new offspring. The procedure offers advantages over the drawing of a blood sample from the infant after birth in certain cases where tendency to bleeding is a very dominant feature.[16] However, it should be borne in mind that admixture of small amounts of maternal blood would, if the latter is normal, give rise to a detectable amount of the enzyme.

SUMMARY

Details of an enzymatic assay for galactose-1-phosphate uridyl transferase in blood are described; the assay is based on the consumption of uridinediphospho-glucose in the presence of excess galactose-1-phosphate. The absence of this enzyme in blood is characteristic of congenital galactosemia, and the test serves to diagnose cases of this disorder.

For valuable technical assistance thanks are due to Mr. Elwood Bynum and to Miss Bodil Waage-Jensen, trainee under the American Scandinavian Foundation through a grant-in-aid made available to one of us (H. M. K.) by the Eli Lilly Laboratories.

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A SPECIFIC ENZYMATIC ASSAY FOR THE DIAGNOSIS OF CONGENITAL GALACTOSEMIA

II. The Combined Test With 4-Epimerase

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THE conversion of the galactose structure into that of glucose in the living cell[1] proceeds according to the following equation: UDPGal[*] $\rightleftharpoons$ UDPG. The enzyme catalyzing this reaction is called UDPGal-4-epimerase[2] and is abbreviated 4-epimerase. (This enzyme has previously been called galacto-waldenase.) 4-Epimerase from mammalian sources requires DPN as a cofactor.[3] As mentioned in the previous article,[4] hemolysates from the human erythrocytes do not catalyze the conversion of UDPGal to UDPG unless DPN is added. This applies to hemolysates from normal as well as from galactosemic individuals. Hence, UDPGal formed by the enzymatic reaction between UDPG and Gal-1-P, catalyzed by the enzyme Gal-1-P uridyl transferase, will accumulate. This makes it feasible to estimate the extent of the reaction by the consumption of UDPG brought about by addition of Gal-1-P. This assay is called the consumption test.[4] It would be desirable to reinforce the reproducibility and specificity of the diagnostic assay for congenital galactosemia by combining the estimation of UDPG consumption with an additional estimation of UDPGal formation brought about by the addition of Gal-1-P. This can be done by adding purified 4-epimerase to the filtrate at the termination of the UDPG estimation. Recent work[5] has made it possible to fractionate and isolate 4-epimerase in sufficiently large amounts to make it feasible, within the near future, to have 4-epimerase commercially available.

METHODS

Stock Solutions.—Stock solutions are identical with those described in the first paper of this series[4] with the addition of UDPGal-4-epimerase. The preparation of this enzyme will be described in a paper to be published shortly.[†5]

Special Equipment.—See paper I of this series, p. 469, this issue.[4]

Procedure.—*Preparation of blood sample, estimation of hemoglobin, incubation of* hemolysates, and deproteinization of the latter are the same as already described.[1]

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[*] The following abbreviations are used: UDPGal for uridinediphosphogalactose, UDPG for uridinediphosphoglucose, DPN for diphosphopyridine nucleotide, Gal-1-P for α-galactose-1-phosphate.

[†] The fractionation of acetone powder from liver for UDPGal-4-epimerase which involves isoelectric precipitation and fractionation with acetone and ammonium sulphate can now be performed with great facility and in a reproducible way. The fractionated enzyme can be lyophilized and kept in this state for months without serious loss of activity. A reprint of the purification procedure can be obtained on request by communication with the authors of the present article.

The determination of the UDPG in the filtrate is also performed exactly as described in the first paper of this series.[4] However, when the density increase after addition of UDPG dehydrogenase has leveled off, 4 epimerase in the amount of 20 μl (containing 100 to 300 units*) is added *first to the control cuvette and subsequently to the cuvette con*taining galactose 1 phosphate. The third cuvette containing inorganic pyrophosphate is not assayed with this enzyme. After the addition of 4 epimerase, the assay mixture is mixed by placing a piece of parafilm on the top of the silica cuvette and inverting it rapidly several times. Readings at 340 mμ are obtained at 30, 60, and 120 seconds after the addition of the enzyme. In order to check for changes in turbidity, the density at 400 mμ is read at 2½ minutes and again at the termination of the assay. The density at 340 mμ is read at various intervals (i e, 5, 10, 15, and 20 minutes). The density at this wave length is followed until the increase has leveled off. This usually requires 40 to 60 minutes. The same routine described for the first cuvette is carried out for the second, which can be started 4 to 6 minutes after the first. The density increase at 340 mμ in *the control should be zero or negligible (occasionally a slight decrease appears in the ex*perimental sample). The number of micromoles of UDPGal formed by interaction of Gal 1 P with UDPG should be equivalent to the amount of UDPG consumed (see Fig 1). The calculation of activity is carried out as described in paper I.[4] Inasmuch as the density increase due to addition of 4 epimerase often proceeds at a slower rate than that caused by addition of UDPG dehydrogenase, the end point may not be as sharp in the second assay period as in the first. Hence, the accuracy of the determination obtained by the 4 epimerase may not be as high as that obtained in the consumption test. If the accuracy of the consumption test is estimated to about ± 10 per cent, that of the 4 epimerase test may be estimated to the order of *± 15 to 20* per cent.

RESULTS

The results obtained using the test described with hemolysates from three normal and one galactosemic adult are shown in Table I. The Δ $D/_{340}$ in the first two columns of the table is due to reduction of DPN by UDPG and the specific dehydrogenase. It is, therefore, a measure of UDPG remaining after incubation of hemolysates and UDPG with or without the addition of Gal-1-P. In the case of normal individuals there is less UDPG remaining, and hence less Δ $D/_{340}$, when Gal-1-P is present during the incubation with hemolysate than when Gal-1-P is not added. This is due to the presence in the hemolysate of the enzyme Gal-1-P uridyl transferase, which forms UDPGal from UDPG and Gal-1-P. Since this enzyme is missing in hemolysates from galactosemic individuals, there is no disappearance of UDPG upon incubation with Gal-1-P. The amount of UDPG remaining, and hence the Δ $D/_{340}$, is the same after

TABLE I ANALYSIS OF FILTRATES BY THE CONSUMPTION TEST AND BY THE COMBINED TEST WITH 4 EPIMERASE

	Δ D/340 WITH DEHYDROGENASE		Δ D/340 WITH 4 EPIMERASE	
SUBJECT	WITH GAL 1 P	WITHOUT GAL 1 P	WITH GAL 1 P	WITHOUT GAL 1 P
1 Normal adult	0.025	0.405	0.340	−0.010
2 Normal adult	0.025	0.395	0.350	+0.010
3 Normal adult	0.130	0.360	0.250	+0.020
4 Galactosemic adult	0.305	0.300	0	0

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*One unit is a density increase at 340 mμ of 0.001 per minute in 0.5 ml with optimal substrate concentration.[5] Since the conditions in filtrates of hemolysate are not optimal, the density increase in the present assay will be 0.020 to 0.040 per minute.

incubation with or without Gal-1-P. The Δ D/$_{340}$ in the last two columns is due to DPN reduced in the same cuvettes after conversion of UDPGal to UDPG by the 4-epimerase. It is, therefore, a measure of UDPGal formed by the enzyme Gal-1-P uridyl transferase upon incubation of hemolysates with UDPG and Gal-1-P. Since hemolysates from normal individuals contain this enzyme, there is UDPGal formed, and hence a high Δ D/$_{340}$, when Gal-1-P is present during the incubation. When Gal-1-P is not added, there is no UDPGal formed and hence no Δ D/$_{340}$. In the case of galactosemic individuals there is no UDPGal formed, and hence no Δ D/$_{340}$ even when Gal-1-P is added. Figs. 1 and 2 are graphic representations of two of the same determinations.

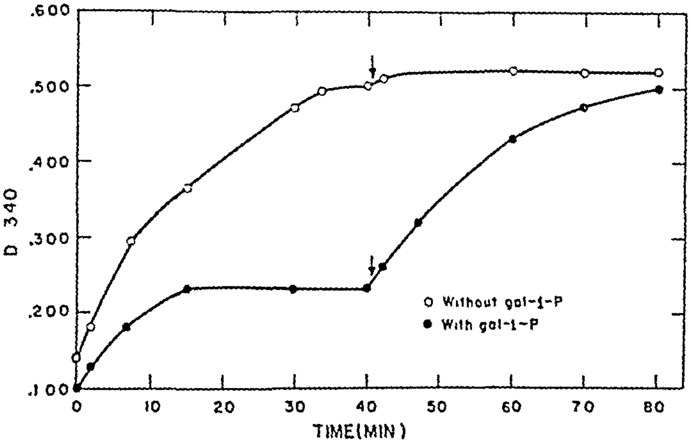

Fig. 1.—Analysis of filtrate of normal adult. UDPG dehydrogenase added at 0 minute; 4-epimerase added at 40-minute point (indicated by arrow).

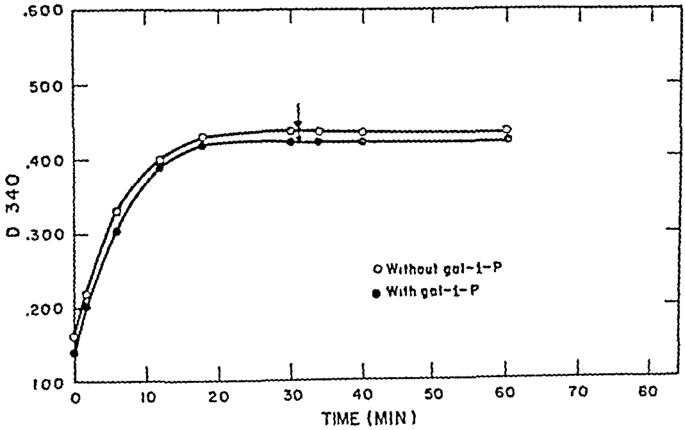

Fig. 2.—Analysis of filtrate in galactosemic adult UDPG dehydrogenase added at 0 minute; 4-epimerase added at 30-minute point (indicated by arrow).

SUMMARY

The first paper of this series described a specific enzymatic assay for the diagnosis of congenital galactosemia. This test was based on the determination of the amount of uridinediphosphoglucose utilized when this uridine nucleotide is incubated with red blood cell hemolysates and galactose 1 phosphate The present paper extends the assay to include not only a measure of uridinediphosphoglucose utilized but also a measure of uridinediphosphogalactose formed during the incubation

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ASPIRATION OF BONE MARROW FROM THE ANTERIOR SUPERIOR ILIAC SPINE

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THE purpose of this article is to describe briefly an apparently new method of bone marrow aspiration which has been highly satisfactory in my hands for the past eight years.

METHOD

The site of puncture is the anterior superior iliac spine. It is located with fingers as an oval protuberance (Fig. 1). Following preparation of the skin and after local anesthesia has been effected, the puncture needle is lodged securely in the periosteum in the middle of this protuberance. The patient should be lying flat, preferably on a firm surface. With the pelvis in this position the needle is pressed into the ileum pointing slightly cephalad (Fig. 2).

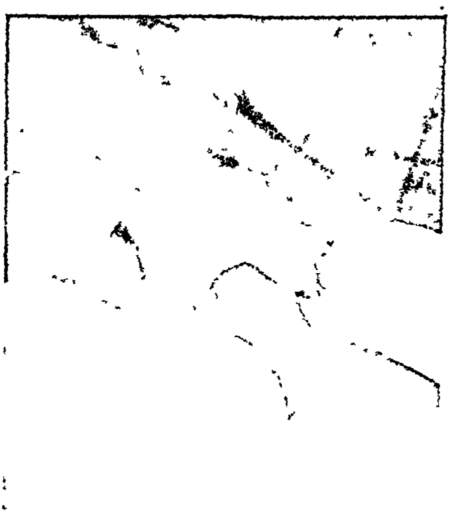

Fig 1

Fig 2.

Fig 1—The oval protuberance of the superior iliac spine is located between the thumbs and forefingers.

Fig. 2—The needle is lodged in the middle of the superior iliac spine pointing slightly cephalad.

In children this method allows for better control of the puncture site. An assistant places one hand on the opposite hip and the other arm on the thighs. A second assistant holds the arms. The operator places the hypothenar eminences of both hands so as to

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From the Laboratory Department of East Tennessee Baptist Hospital, Knoxville, Tenn.
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exert pressure for control on the hip. The needle can be lodged with the thumb and forefinger. Pressure is then applied with one hand while control of the hip is maintained with the other.

DISCUSSION

The superior iliac spinal puncture has been easier than sternal puncture in my hands This has been in a large part due to increased cooperation of the patient. There is considerable apprehension by the patient when a large needle is introduced into his chest just over the heart. This apprehension is often shared by the operator, particularly when he seldom performs bone marrow aspiration While the incidence of injury of underlying structures has not been great in sternal puncture, it is nevertheless a possibility.[1]

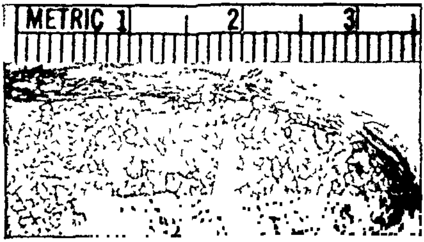

Fig 3—Longitudinal section of the superior iliac spine showing thinner compact bone at the site of puncture indicated by arrow

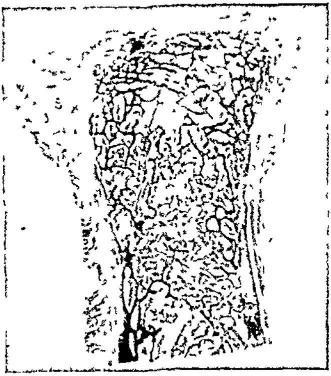

Fig 4—Cross section of the superior iliac spine at the site of puncture showing the compact bone of the lateral wall of the ilium

The iliac spine is a readily accessible area. Its movement relative to the puncture can easily be controlled by the technique described. This is particularly important in obtaining bone marrow from children Firm pressure may be applied to the needle when lodged in the iliac spine without trepidation. Since the bone of the spine is relatively porous, it will not allow the needle to slip off into the soft tissues, once it has been correctly lodged. In case the needle should suddenly puncture through an easily yielding cortex, as occasionally happens in bone which has been altered by hyperplastic bone marrow, the momentum can carry the needle point to only a dense, oblique, iliac wall cortex.

Microscopic study of the superior iliac spinal bone structure indicates that it is particularly favorable for marrow aspiration. The cortical bone density over the spine at the site of puncture is less than over the iliac crest (Fig. 3) and is considerably less as compared to the lateral wall of the ilium (Fig. 4). It is anatomically advantageous to lodge the needle in the middle of the iliac spine and to introduce it with a 15 degree cephalad direction as described, but considerable margin of deviation from the 15 degree angle will be satisfactory. When the needle is lodged exactly in the center of the iliac spine, even extreme angles will allow the needle to enter the marrow space.

The iliac bone marrow has been satisfactory for diagnostic purposes. Duplicate studies, when done with sternal marrow specimens, have indicated no variations. This has also been the experience of others.[2] Occasional dilution of specimens was encountered, but inadequate or unsatisfactory specimens were confined to cases of bone marrow hypoplasia and similar conditions, which were subsequently demonstrated by repeated aspiration at several sites, and marrow biopsy.

SUMMARY

Aspiration of bone marrow from the anterior superior iliac spine is described and discussed as having advantages over the methods generally in use.

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A FLUOROMETRIC METHOD FOR TOTAL SERUM CHOLESTEROL

D B McDougal, Jr, M D,* and H S Farmer
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A METHOD for serum cholesterol was needed for the rapid analysis of the small amounts of blood available from the tip of a rat's tail A simple method was developed which requires 4 μl of serum or less Aside from its high sensitivity, the method offers certain advantages in ease of manipulation over methods currently in use in clinical laboratories[1-3] It was thought therefore, that the method might be of interest to those concerned with clinical estimation of cholesterol The procedure is based on a fluorometric method previously published[4]

MATERIALS AND EQUIPMENT

Acetic acid, acetic anhydride, and sulfuric acid meeting A C S specifications for analytic reagents may be used without further purification 1,1,2 Trichloroethane† is purified as follows[4] it is distilled, the fraction boiling at 111° to 113° C is collected, washed 4 times with 0 1 volume of concentrated sulfuric acid, twice with water, and dried over anhydrous sodium sulfate Cholesterol standard solutions (100 to 400 mg per cent) are prepared in trichloroethane

Lang Levy constriction pipettes[5, 6] are used for volumes less than 1 ml For concentrated sulfuric acid, because of the high viscosity, the tip and constriction need to be rather wide

Fluorometer tubes are selected from 3 ml (10 × 75 mm) Pyrex test tubes They are cleaned by rinsing twice with a detergent solution (Dreft Procter and Gamble other detergents may be as good or better but have not been tested) and 4 times with distilled water

PROCEDURE

Four microliters of serum are added to 40 μl of a 3 2 mixture (V/V) of glacial acetic acid and trichloroethane in 10 × 75 mm tubes A precipitate forms, but will dissolve with thorough mixing (if care is not taken to dissolve the precipitate at this point the results will be erratic and low) To the clear solution, 1 ml of a freshly prepared mixture of trichloroethane and acetic anhydride 5 1 (V/V) is added by syringe pipette‡ and mixed 40 μl of concentrated sulfuric acid are then added with immediate and thorough mixing §

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§Rapid efficient mixing is easily obtained by buzzing[7] which eliminates the need for the introduction of any implement into the solution This may be accomplished with simple equipment in use in this laboratory for many years It is believed that buzzing would save time and equipment in many laboratory operations and merits a brief description Tubes of 2 to 10 mm diameter are held firmly against a steel cone mounted on the shaft of a small motor (1/25 H P 5 000 r p m Type NSE 11 Bodine Electric Company Chicago No 2168 1950) The cone 2 in in over all length is turned from a ½ in steel rod The taper occupies 1½ in of the over all length A 5/16 in hole ½ in deep is drilled in the large end at an angle of about 2° with the axis (The tip of the cone is therefore about 1/16 in off center whereas the base is nearly on center) The speed of the motor is governed by a variable transformer (Type 20 Powerstat Superior Electric Company Bristol Conn) the smaller the tube the higher the speed required Tubes of 10 to 18 mm diameter may be stirred equally well with a larger motor (¼ H P 1 750 r p m) fitted with a slightly bent steel rod No loss of contents occurs provided the tube is no more than one third to two fifths full

The mixture is centrifuged, and the clear supernatant solution is poured into fluorometer tubes. The samples are read using Corning filters Nos. 4010 and 5120 for the primary, isolating the 546 mµ mercury line, and No. 2424 (a cut-off filter with 37 per cent transmittance at 590 mµ) for the secondary. Solutions of 0.01 to 0.1 mg. per cent safranine O* or rhodamine B† in 0.01 N HCl *may be used in the reference tube to set the fluorometer.* At least forty minutes are allowed to elapse between the addition of sulfuric acid and reading. Thereafter time is not critical, but after several hours two phases separate and readings are no longer accurate.

To the blanks, 4 µl of water are added instead of serum. Standards are prepared by adding 4 µl of water and 5 to 40 µg of cholesterol in 5 to 10 µl of trichloroethane to the acetic acid–trichloroethane mixture. At final concentrations of cholesterol above 5 µg per ml., fluorescence is not proportional to the concentration and calculation may be made from a standard curve.

If serum is not limited and pipetting 4 µl seems difficult, a larger volume of serum may be diluted with 10 volumes of the acetic acid–trichloroethane mixture. A 40 µl aliquot of this may be added to 1 ml. of the trichloroethane-acetic anhydride mixture, and the analysis completed as described.

TABLE I. CHOLESTEROL RECOVERY BY PROPOSED METHOD

SERUM CHOLESTEROL		CHOLESTEROL ADDED	CHOLESTEROL RECOVERED	
MG. %	µG IN SAMPLE	(µG)	(µG)	(%)
214	9.05	6.12	5.88	96
			5.88	96
			5.45	89
			5.55	91
198	8.36	6.12	5.47	89
			5.76	94
			5.76	94
			5.97	98
163	6.88	6.12	5.97	98
			5.77	94
			6.45	105
			6.45	105
266	11.23	6.12	5.35	88
			5.54	90
			5.77	94
			5.54	91
269	11.36	15.35	15.84	103
			15.72	102
			14.86	97
			15.09	98
507	21.40	15.35	14.37	94
			13.95	91
			14.30	93
			14.47	94
338	14.23	15.35	14.60	95
			15.79	103
			14.70	96
			15.02	98
Average				96

COMMENTS ON THE METHOD

The initial dilution of serum with the acetic acid–trichloroethan mixture permits subsequent addition of a large volume of trichloroethane–acetic anhydride without trapping cholesterol in the precipitate. Without this preliminary step, the results are invariably low When 5 to 17 volumes of acetic acid–trichloroethane are mixed with 1 volume of serum a clear solution results With less than 5 or more than 17 volumes the mixture is cloudy

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*National Aniline Division, New York, N. Y.

†Distillation Products Industries, Rochester, N. Y.

The concentration of sulfuric acid used is optimal With lower concentrations, development of fluorescence is slower and incomplete With higher concentrations, two liquid phases are present

After the addition of sulfuric acid the precipitate may stick to the walls of the tube above the solvent level This has no effect on the precision of the results

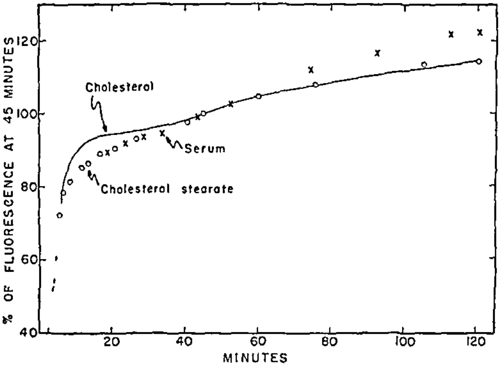

Fig 1—Development of fluorescence with time for cholesterol cholesterol stearate and serum extract. The tubes contained 6 88 μl of cholesterol solution (4 mM 155 mg per cent) or cholesterol stearate (4 mM) or 4 22 μl of serum (270 mg per cent) in a final volume of 1 09 ml Water (4 μl) was added to cholesterol and cholesterol stearate tubes The actual readings at 45 minutes were cholesterol 75 0 cholesterol stearate 75 6 and serum 90 7 (interpolated)

RESULTS

After the initial rapid development of fluorescence, there is a slow rise (about 20 per cent per hour, Fig 1) However, since the percentage change in fluorescence with time is about the same for the samples and the standards, this rise does not affect the results if the tubes are read in the order in which sulfuric acid was added, and the readings may be made at leisure

There is little difference between the fluorescence developed from cholesterol and cholesterol stearate on an equimolar basis, and what difference there is disappears after 30 minutes (Fig 1)

Since substances other than cholesterol will interfere with this method only to the extent that they do with the method of Albers and Lowry,[4] it is calculated that the hormonal steroids and bile salts present in blood contribute less than 3 mg per cent to the final apparent cholesterol level

Recovery experiments were performed, and the results are given in Table I Recovery of added cholesterol averaged 96 per cent It is uncertain whether recovery by other methods may not also be incomplete since it is difficult in those methods to introduce cholesterol in a valid manner

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The concentration of sulfuric acid used is optimal. With lower concentrations, development of fluorescence is slower and incomplete. With higher concentrations, two liquid phases are present.

After the addition of sulfuric acid the precipitate may stick to the walls of the tube above the solvent level. This has no effect on the precision of the results.

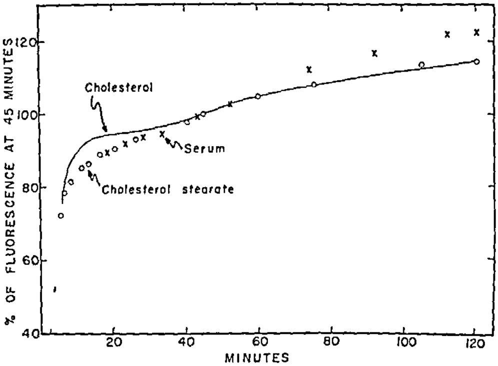

Fig. 1.—Development of fluorescence with time for cholesterol, cholesterol stearate and serum extract. The tubes contained 6.88 µl of cholesterol solution (4 mM, 155 mg per cent) or cholesterol stearate (4 mM) or 4.22 µl of serum (270 mg per cent) in a final volume of 1.09 ml. Water (4 µl) was added to cholesterol and cholesterol stearate tubes. The actual readings at 45 minutes were cholesterol 75.0, cholesterol stearate 75.6 and serum 90.7 (interpolated).

RESULTS

After the initial rapid development of fluorescence, there is a slow rise (about 20 per cent per hour, Fig. 1). However, since the percentage change in fluorescence with time is about the same for the samples and the standards, this rise does not affect the results if the tubes are read in the order in which sulfuric acid was added, and the readings may be made at leisure.

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those methods to introduce cholesterol in a valid manner.

The results obtained with the new method were compared with those obtained by the method now in use in Barnes Hospital*[1] and with those obtained by the method of Albers and Lowry.[4] The correspondence was good (Table II).

TABLE II. CHOLESTEROL VALUES OBTAINED BY THREE DIFFERENT METHODS

SERUM NUMBER	SAIFER AND KAEMMERER[1] (MG. PER CENT)	ALBERS AND LOWRY[4] (MG. PER CENT)	PRESENT METHOD (MG. PER CENT)
1	217	206	209
2	157	187	187
3	158	154	160
4	272	250	268
5	380	331	322
6	333	323	333
7	245	254	262
Average	280	272	277

DISCUSSION

The present method appears to be simpler than methods now in use for serum cholesterol. Thirty to sixty samples may be run in three hours or less, and the samples are $1/25$ to $1/125$ the size of those required by other methods. No attempt has been made to develop maximum sensitivity, but a ten to twenty fold increase could doubtless be obtained merely by a corresponding reduction in the volumes used.

The method has been used for repeated determinations on the sera of rats with satisfactory results. It has also been successfully applied to rat and human liver homogenates.†

SUMMARY

A simple, rapid, accurate fluorometric method for the assay of total serum cholesterol is described requiring as little as 4 μl of serum. Sixty analyses may be run in less than three hours.

Grateful acknowledgment is made to Drs. O. H. Lowry and E. Robins for help and encouragement.

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*We are indebted to Dr. H. M. Perry of the Department of Medicine for these determinations.

†Personal communication, Dr. H. B. Burch.

A RAPID METHOD FOR PREPARATION OF PLASMA PROTEINS FOR RADIOASSAY

JOSEPH KATZ, PH.D., ALVIN L. SELLERS, M.D., AND JESSIE MARMORSTON, M.D.
LOS ANGELES, CALIF.
WITH THE TECHNICAL ASSISTANCE OF SHIRLEY RAUCH, B.A.

THE use of plasma proteins labeled with radioisotopes in metabolic studies is quite extensive. For beta ray assay, two general methods of sample preparation have been used. In one, the purified fractions are combusted to carbonate or sulfate.[1] In the other, the proteins are converted to fine dry powders.[2] Both methods are slow and tedious, the first requires combustion and the second requires repeated washings and centrifugations.

A simple and rapid method is described here for preparing plasma protein for beta ray assay. *Protein is flocculated by hot trichloroacetic acid and the precipitate plated on filter paper.* Another procedure using protein mounting on filter paper has also been described by Armstrong and associates.[3]

MATERIALS AND METHODS

Reagents.—Three and ten per cent solutions of trichloroacetic acid, acetone, and acetone ethyl ether (1:1) are employed.

Materials.—Whatman No. 540 filter paper. This is available in diameters of 2.1, 2.4, and 3.7 cm. All these sizes are used in this laboratory and the choice depends on the amount of protein (see below) and the type of Geiger tubes available. Most commercially available thin mica window tubes have an internal diameter of about one inch. The diameter of the protein precipitate is 4 to 6 mm. smaller than that of the paper, therefore, with these tubes, only the two smaller sizes of paper can be used. With metalized plastic window tubes of a larger internal diameter, use of the 3.7 cm. paper is advantageous and permits the use of larger amounts of protein.*

The filtering device for use with 2.4 cm. paper is shown in Fig. 1.† A coarse sinter and well ground surfaces are important. For efficient operation two such filters are used simultaneously.

Procedure.—An aliquot of plasma, serum, or protein solution containing 5 to 60 mg. of protein (see below) in a 20 × 150 ml. tube is diluted with 0.9 per cent sodium chloride solution to 5 ml. Ten milliliters of 10 per cent trichloroacetic acid are added and the tubes heated for 10 minutes in a gently boiling water bath.

To assay plasma albumin, aliquots of plasma in plastic tubes are diluted to 1.0 ml. with 0.9 per cent sodium chloride, 5 ml. of 33 per cent ammonium sulfate is then added. The

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*Covered plastic holders in book form containing 20 to 25 papers facilitate greatly the handling and storage of the samples. These are available from Research Specialties Co., 2005 Hopkins Avenue, Berkeley, Calif.

†The filters can be ordered from California Glassblowing Co., 35 South Raymond Avenue, Pasadena, Calif.

precipitates in the medium range. Indeed, in the 10 to 20 mg. range, even with a large number of samples, the total error does not greatly exceed the statistical error of counting.

The self-adsorption with the hard beta radiation of I^{131} is small and, as seen in Table 1, within a 10 mg. range it is not significant. By selecting a working range of 8 to 16 mg. of protein, we have avoided self-adsorption corrections altogether.

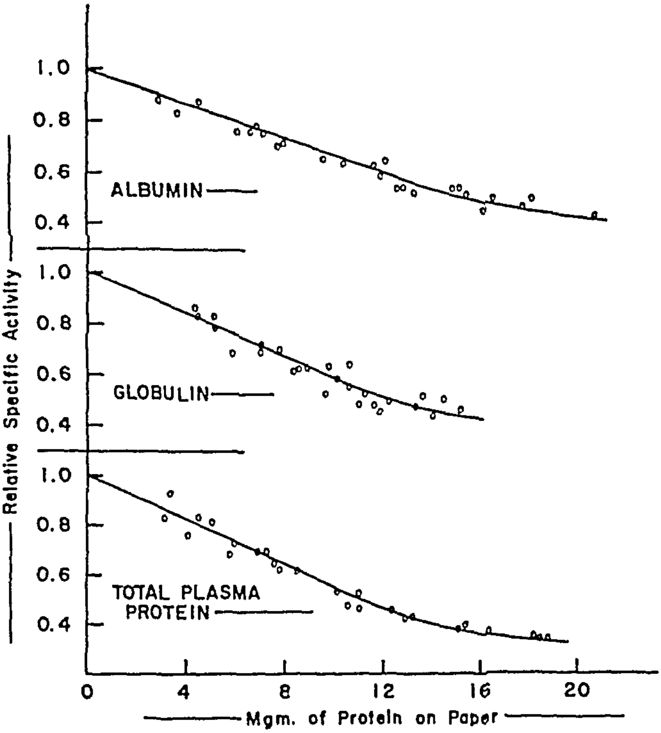

Fig. 2.—Self-absorption of C^{14}-labeled rat plasma proteins. Proteins precipitated on 2.4 cm. Whatman No. 540 filter paper. The area of precipitate is 3.1 sq. cm. Activity was assayed with a gas flow, plastic window tube. The extrapolated specific activities for 0 mass were as cts./mg./min., 38 for total plasma protein, 51 for albumin, and 14 for globulin. Error of counting ±5 per cent.

Results obtained with C^{14}-labeled rat plasma proteins are presented in Fig. 2 where the specific activity is plotted as a function of sample weight.

The specific activities decrease linearly until 12 mg. beyond that the slopes flatten out markedly. In the linear range, the self-adsorption of the globulin and total plasma protein precipitates was about the same but larger than that of albumin. Thus, when the extrapolated specific activity at 0 mass is taken as 1, the observed specific activity at 10 mg. (3.2 mg. per square centimeter) was 0.55 and 0.57 for total protein and globulin, but 0.66 for albumin. The optimal weight range is from 6 to 10 mg. requiring about 0.1 to 0.2 ml. of plasma for total protein, and twice this for globulin and albumin assays.

With rats, the amount of blood that can be drawn repeatedly is limited, but when larger amounts of plasma are available the use of larger papers, thus increasing the working range, is preferable

The method described here does not have the precision attainable when samples are combusted and assayed as $BaCO_3$, however, it has the advantage of speed and is sufficiently precise for many purposes

SUMMARY

A simple and rapid technique for radioactivity assay of isotopically labeled plasma proteins is described The plasma proteins are flocculated by hot trichloroacetic acid and mounted on filter paper disks Results obtained with I^{131} and C^{14} labeled plasma proteins are described

ADDENDUM*

Recently we have found glass fiber filter paper useful for mounting precipitates This paper (made by Hurlbut Paper Co, No X—934—Ah, distributed by Reeve, Angel and Co) is available in 24 mm and 37 mm sizes Although more retentive, it filters much faster than Whatman 540 or 542 paper, it is lighter and tends less to curl

For assay, the papers are put in regular shallow aluminum planchets and are held in place with a flat brass washer

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3 Armstrong S H, Mcleod, K, Wolter, J, and Kukral, J The Persistence in Blood of the Radioactive Label of Albumin, Gamma Globulins, Globulins of Intermediate Mobility Studied With S^{35} and Paper Electrophoresis, J LAB & CLIN MED 43 918, 1954

Erratum

In the article by David Platt, M S, Howard L Holley, M D, and Ward Pigman, Ph D, entitled "Changes in the Electrophoretic Behavior of Arthritic Synovial Fluid Components Following Intra articular Steroid Therapy," which appeared in the May issue of the JOURNAL, pages 762 766, the last paragraph on page 763 should read as follows

"Total nitrogen expressed as protein (g nitrogen × 6 25) in the active state averaged 5 0 ± 1 1 (S D) g per 100 ml and nondialyzable nitrogen × 6 25 averaged 4 6 ± 1 1 g per 100 ml Upon improvement, the average total nitrogen × 6 25 was 4 9 ± 1 6 g per 100 ml and the average nondialyzable nitrogen × 6 25 was 4 5 ± 1 7 g per 100 ml "

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*Note added in proof

A SHORT ALBUMIN METHOD FOR THE DEMONSTRATION OF ISOHEMAGGLUTININS, PARTICULARLY INCOMPLETE Rh ANTIBODIES

Marion Lewis and Bruce Chown, M.D.
Winnipeg, Canada

THE method here described, which we have used for several years, developed out of one we published in 1948.[1] It differs from those described in standard manuals[2-5] and has advantages over them: it is rapid and in our hands, using undiluted serum alone, it never misses a so-called zone Rh antibody. Furthermore it will detect weak Rh antibodies, even, in a rare case, picking up such an antibody that does not react by the saline, trypsin, or indirect Coombs methods.

No one method will detect all antibodies; the present one will pick up anti-A, B, O or H, M, S, s, P, K, k, Le^a, Le^b, the Rh antibodies and some of the rare ones. In our hands no albumin method has been successful with all anti-Fy^a antibodies; for them the indirect Coombs method is necessary.

METHODS

I. *When Several Sera Are To Be Tested With Any One Blood Sample.*—

1. Wash the cells 3 times in saline. Spin to pack, and remove the remaining saline as completely as possible with a Pasteur pipette.

2. One drop of an albumin-red-cell mixture is to be used with each serum under test (and each dilution of each serum that is to be titrated). Of 30 per cent bovine albumin,* place, to the nearest 10 drops, the number of drops so needed in a small test tube, and for every 10 drops of albumin add 1 drop of the washed, packed red cells. Mix well.

3. With a serologic pipette place 1 drop of each serum under test in a test tube 80 by 6 mm. internal diameter, followed by 1 drop of the albumin-red-cell suspension. Mix vigorously. Let stand for 10 minutes at room temperature using an automatic timer. Spin at 1,500 r.p.m. for 2 minutes. The exact timing is most important both for the original "incubation" and for the centrifuging.

4. The reaction may be read at once or may be left for reading at a later convenient time. Readings are made macroscopically by rotating the tube gently until all cells are freed from the bottom, and then gently tilting them up and down along the side of the tube. The readings are as follows:

S	one solid clump with a smooth constant edge;
S⁻	one clump that does not retain a smooth edge;
++++	2 or 3 large clumps in clear fluid; or one clump that breaks into 2 or 3 on tilting;
+++	several large clumps in clear fluid;
++	small clumps in clear fluid;
+	distinct evenly distributed fine clumps (like brick dust);
-	no clumps visible to the naked eye.

When dealing with an unknown serum all reactions are read microscopically (with a known Rh antibody, the solid to ++++ reactions would not be). Take a glass rod about 4 mm.

From the Blood Group Reference and Research Laboratory, Children's Hospital Postal address: 735 Notre Dame Ave., Winnipeg 3, Manitoba

Received for publication March 29, 1957.

*We have found Armour's satisfactory.

in diameter by 10 cm long, dip it in saline and place a small drop on a slide (As many as 18 drops, 9 in each of two rows, can be placed on one slide) Bounce the rod in a cell albumin serum mixture Some cells adhere to the rod, mix these in one of the drops of saline and spread gently With good eyesight the macroscopic and microscopic readings should agree down to a + reading Beyond this one may read a weak + and a trace reaction microscopically, the latter no more than an uneven appearance of the cell distribution These are recorded, respectively, as ∓ and $\overline{\text{tr}}$, the upper bar being the macroscopic and the lower the microscopic reading

The above is our standard method when a number, say 30 to 50, Rh negative pregnancy bloods are being tested on one day and an average amount of serum is available The test cells are selected to include all known antigens, at least 2 different test cells being used Mixtures of test cells are not used because of the difficulty thereby encountered in reading very weak reactions

II *When Only a Small Volume of Serum Is Available*—

A *To test the serum against a single blood sample*

1 Wash cells as above, removing the final saline as completely as possible

2 Using a fine Pasteur pipette, place first in the bottom of a test tube of 50 by 5 mm internal diameter 1 small, measured drop of serum, and then 1 drop of equal size of 30 per cent bovine albumin (The drop used is roughly 0 02 ml The tip of the pipette is marked with a grease pencil at the level to contain this amount)

3 Rinse the pipette and dip the tip in the concentrated red cells Take up a volume equal to approximately one fifth of the volume of serum and place it in the serum albumin mixture

4 Mix thoroughly, flicking if necessary to get rid of any bubbles

5 Let stand for 10 minutes at room temperature, centrifuge at 1500 r p m for 1¼ minutes and read macroscopically as above For the microscopic reading take a drop of the sediment up in a Pasteur pipette, place it in a drop of saline on a slide, and draw the mixture gently across the slide, making a pool approximately 2 3 by 10 mm

B *To test a serum against a large panel of cells*

1 Add to the required amount of serum an equal voluume of 30 per cent bovine albumin Mix thoroughly The portion of this mixture not used at once may be stored frozen

2 Wash red cells as above, removing all supernatant

3 Place 2 measured drops (as above) of the serum albumin mixture in the small test tube

4 Add red cells as in *IIA* and proceed as there outlined

III *Titration of an Albumin Active Antibody*—Twofold serial dilutions of the serum under test are made in normal saline, and the tests are then carried out as above

Examples of Use With High Titer and Zone Antibodies—We have long been satisfied with this method for demonstrating the presence, specificity, and titer of albumin active, particularly Rh, antibodies Nevertheless, we thought it desirable to compare our method in our hands with the standard National Institutes of Health method in the hands of someone expert in the use of the latter in this Dr Neva Abelson kindly cooperated, we are most grateful to her.

Dr Abelson sent us the following five sera

No 271 From a sterilized woman who had been stimulated to further antibody production

No 317 From a man with a transfusion history, whose antibody had been stimulated over a period of time

No 307 From a woman whose antibody was completely missed in another laboratory because of a zone

DIRECT TITRATION OF CALCIUM IN BLOOD SERUM

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CHARLES N. REILLEY, PH.D.**
CHAPEL HILL, N. C.

THE direct titration of calcium in blood serum would save substantial amounts of time in laboratories now using the classic Clark and Collip[1] modification of the Kramer and Tisdall[2] method or the method as later modified by Tisdall.[3] A direct titration has been described by Elliott[4] using disodium ethylenediaminetetraacetate (EDTA) as a complexometric titrating agent with ammonium purpurate as an indicator. This indicator has certain disadvantages. In addition to its instability it has been the experience of the authors and other workers in these laboratories that the pink to purple color change is difficult to judge. In the presence of magnesium, as Hildebrand and Reilley[5] point out, this color change is even more gradual and indefinite. Because of these difficulties the latter authors[5] proposed the use of a new indicator (Calcon) for the titration of calcium.

Calcon, 1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulfonic acid, CI202, produces a blue color between pH values of 7.4 and 13.5 in aqueous solutions. Below a pH of 7.4 or above a pH of 13.5 the indicator is pink. At a pH of approximately 13, where magnesium is precipitated as the hydroxide, only calcium ions react with the indicator to form a pink-colored complex. One may then titrate the calcium directly with EDTA, the end point being detected by a color change from pink to pure blue.

The procedure proposed herein uses this indicator to detect the end point in the direct EDTA titration of calcium in serum. It is rapid, requires only one milliliter of serum, and the color change from pink to pure blue is reasonably distinct. Furthermore calcium may be determined directly in serum without prior precipitation and washing.

MATERIALS AND METHODS

Indicator Solution.—Fifty milligrams of Calcon† are dissolved in 10 ml. of methanol. This solution is stable for several weeks at room temperature.

Sodium Hydroxide 2 M.—Eighty grams of NaOH‡ are dissolved in distilled water and diluted to one liter.

EDTA Solution 0.0500 M.—Approximately 3.7 Gm. of disodium ethylenediamine tetraacetate dihydrate† are dissolved in 200 ml. of distilled water. This solution is standardized by the procedure outlined below.

Standard Calcium Solution.—Primary standard grade calcium carbonate§ is dried in an oven overnight at 110° C. Exactly 0.1001 Gm. is transferred to a 100 ml. volumetric flask

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†J. T. Baker Chemical Company, Phillipsburg, N. J.

‡Reagent, Merck & Co., Inc., Rahway, N. J.

§Mallinckrodt Chemical Works, St. Louis, Mo.

with about 15 ml. of distilled water. Two milliliters of 1 M HCl are added to the flask which is then heated on a hotplate to about 60° C. to insure complete solution and evolution of carbon dioxide. The inside of the flask is rinsed down with small volumes of distilled water while the solution is still hot. After cooling to room temperature, the contents of the flask are diluted to the mark with distilled water. One milliliter of this stock standard contains 0.40 mg. calcium per milliliter. The working standard, containing 0.10 mg. calcium per 5 ml., is made by diluting 5 ml. of the stock standard to 100 ml. in a separate volumetric flask.

Standardization of EDTA Solution.—Five milliliters of the working standard (0.10 mg. calcium) are pipetted into a 50 ml. beaker, 4 ml. of 2 M NaOH are added, and the mixture is diluted to about 30 ml. with distilled water. Two drops of indicator solution are added and the solution is titrated with the 0.05 M EDTA to a pure blue end point. A "Syringe Microburet"* calibrated to deliver 0.2 μl per scale division is used. The solution is stirred continuously during the titration with an electric stirrer fitted with a small glass paddle.

The molarity of the EDTA solution is calculated by the formula:

$$\text{Molarity EDTA} = \frac{0.0025}{\text{ml. EDTA}}$$

A 5 ml. buret calibrated to 0.02 ml. may also be used, in which case the 0.05 M EDTA solution is diluted 1:50 (0.001 M) prior to standardization. The same formula is used to calculate the molarity of the EDTA.

Standardized EDTA should be stored in Pyrex or polyethylene bottles which have previously been rinsed with a hot alkaline 1 per cent EDTA solution followed by a thorough rinse with distilled water.

Determination of Calcium in Serum.—One milliliter of serum is pipetted into a 50 ml. beaker, 4 ml. of 2 M NaOH added, and the mixture diluted to about 30 ml. with distilled water. Two drops of indicator solution are added, and the solution is titrated rapidly and deliberately to a blue end point with the standard EDTA solution. Since serum may impart varying degrees of a slight yellow component to the titration, the end point may exhibit a slightly greenish tint. This color can be quickly appreciated by adding a definite excess of EDTA to several trial titrations. The true end point is reached when addition of excess EDTA causes no further color change.

The concentration of calcium in serum is calculated from the following formula:

$$\text{Ml. EDTA} \times \text{Molarity EDTA} \times 4{,}000 = \text{mg. calcium per 100 ml. serum}$$

RESULTS

Table I shows a comparison between serum calcium values obtained by the proposed procedure and the method of Tisdall.[3] The latter procedure was used on this group of sera by the Clinical Biochemistry Laboratory of the North Carolina Memorial Hospital, Chapel Hill, N. C.

Duplicate rather than average values are presented to illustrate the reproducibility in each method. The average difference between duplicate titrations within each method itself is 0.23 mg. calcium per 100 ml. serum in the group of data shown and the average difference between the two methods is 0.25 mg. calcium per 100 ml. serum.

The results in Table II are mean values of duplicate determinations by the present method of five serum samples to which 10.0 mg. calcium per 100 ml. serum have been added. The average recovery of added calcium is 10.0 mg. per 100 ml. of serum.

A series of determinations carried out on 10 identical 1.0 ml. samples of pooled serum yielded a mean value of 10.5 with a standard deviation of ±0.15 mg. calcium per 100 ml. serum.

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*Micro Metric Instrument Co., Cleveland, Ohio.

TABLE I. COMPARISON OF PROPOSED METHOD WITH THE PROCEDURE OF TISDALL

SAMPLE NO.	CALCIUM (MG. PER 100 ML. SERUM) PROPOSED METHOD	CALCIUM (MG. PER 100 ML. SERUM) METHOD OF TISDALL[3]
1	10.4, 10.1	9.6, 10.0
2	6.6, 6.2	6.2, 6.0
3	10.0, 9.7	9.4, 9.8
4	12.3, 12.5	12.2, 12.3
5	9.3, 9.4	9.4, 9.4
6	7.2, 7.1	6.2, 6.5
7	9.5, 9.5	9.4, 9.4
8	10.2, 9.8	9.6, 10.0
9	9.9, 9.9	10.0, 10.0
10	10.3, 10.1	10.4, 10.3
11	9.3, 9.2	9.0, 9.8
12	8.7, 8.6	8.8, 8.6
13	7.2, 7.1	6.5, 6.7
14	10.5, 10.3	10.0, 10.3
15	9.9, 9.7	9.9, 9.7
16	6.4, 5.9	6.3, 6.4
17	7.4, 7.8	8.0, 8.1
18	10.0, 10.3	10.2, 9.9
19	*8.9, 8.5*	*9.1, 9.5*
20	8.5, 8.8	8.2, 8.5
21	9.8, 9.7	10.3, 10.1
22	10.8, 11.1	10.7, 10.9
23	10.4, 10.1	10.6, 10.3
24	9.2, 9.0	9.8, 9.7

TABLE II. RECOVERY OF CALCIUM ADDED TO SERUM

SAMPLE NO.	CALCIUM (MG. PER 100 ML. SERUM) SERUM	SERUM, WITH 10.0 MG. CALCIUM ADDED PER 100 ML.	CALCIUM RECOVERED
1	9.4	19.4	10.0
2	12.4	22.3	9.9
3	11.0	21.1	10.1
4	7.2	17.4	10.2
5	8.9	18.9	10.0
Mean			10.0

SUMMARY

A direct titration method for the rapid determination of calcium in serum is presented. The results obtained by this method indicate a degree of accuracy and reproducibility closely approximating that of the classic permanganate titration of precipitated calcium oxalate. The average time required for duplicate titrations is less than 5 minutes.

We wish to thank Dr. C. R. Spell for furnishing the serum samples with their analysis by the permanganate method.

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4. Elliott, W. E.: Volumetric Determination of Calcium in Blood Serum, J. Biol. Chem. 197: 641-644, 1952.
5. Hildebrand, G. P., and Reilley, C. N.: New Indicator for Complexometric Titration of Calcium in Presence of Magnesium, Anal. Chem. 29: 258-264, 1957.

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CLINICAL AND EXPERIMENTAL

A COMPARISON OF SHOCK DUE TO ENDOTOXIN WITH ANAPHYLACTIC SHOCK

MAX H WEIL, M D, AND WESLEY W SPINK, M D
MINNEAPOLIS MINN

THE cellular component of gram negative bacteria, designated as endotoxin, is a lipoprotein carbohydrate complex situated on or near the surface of the bacterial cell[1, 2] This constituent is one of the features that distinguishes gram negative bacteria from gram positive organisms There is no evidence that invasiveness or virulence of the bacterial cell is closely related to the surface endotoxin, but the dissemination in the body of endotoxin from gram negative bacteria having attenuated virulence can provoke a serious, and even fatal, illness Not infrequently, bacteremia due to gram negative organisms has caused irreversible shock[3-7] The increasing clinical significance of shock due to endotoxin has led to extensive inquiry into the underlying mechanisms involved[8-10] In cooperation with the Departments of Physiology and Surgery, the mechanism of this type of shock has been studied intensively and the results have been reported[11-18]

During the progress of the investigations on the mechanism of shock induced in the dog by endotoxin, it became increasingly apparent that the type of shock produced by a single intravenous injection of endotoxin—designated as anaphylactoid shock—was remarkably similar to the reported sequence of findings, known as anaphylactic shock, in sensitized dogs given a second injection of specific antigen These similarities were of considerable interest because of studies in this clinic on the role of brucella endotoxin in the pathogenesis of illness Experimental and clinical observations have indicated quite clearly

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12	8.7, 8.6	8.8, 8.6
13	7.2, 7.1	6.5, 6.7
14	10.5, 10.3	10.0, 10.3
15	9.9, 9.7	9.9, 9.7
16	6.4, 5.9	6.3, 6.4
17	7.4, 7.8	8.0, 8.1
18	10.0, 10.3	10.2, 9.9
19	8.9, 8.5	9.1, 9.5
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CLINICAL AND EXPERIMENTAL

A COMPARISON OF SHOCK DUE TO ENDOTOXIN WITH ANAPHYLACTIC SHOCK

Max H Weil, M D, and Wesley W Spink, M D
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THE cellular component of gram negative bacteria, designated as endotoxin, is a lipoprotein carbohydrate complex situated on or near the surface of the bacterial cell[1, 2] This constituent is one of the features that distinguishes gram negative bacteria from gram positive organisms There is no evidence that invasiveness or virulence of the bacterial cell is closely related to the surface endotoxin, but the dissemination in the body of endotoxin from gram negative bacteria having attenuated virulence can provoke a serious, and even fatal illness Not infrequently, bacteremia due to gram negative organisms has caused irreversible shock[1, 7] The increasing clinical significance of shock due to endotoxin has led to extensive inquiry into the underlying mechanisms involved[8, 10] In cooperation with the Departments of Physiology and Surgery, the mechanism of this type of shock has been studied intensively and the results have been reported[11, 18]

During the progress of the investigations on the mechanism of shock induced in the dog by endotoxin, it became increasingly apparent that the type of shock produced by a single intravenous injection of endotoxin—designated as anaphylactoid shock—was remarkably similar to the reported sequence of findings, known as anaphylactic shock, in sensitized dogs given a second injection of specific antigen These similarities were of considerable interest because of studies in this clinic on the role of brucella endotoxin in the pathogenesis illness Experimental and clinical observations have indicated quite clea

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that illness arises not only because of the nonspecific, intrinsic toxicity of the brucella endotoxin, but also because of the appearance of specific hypersensitivity of host tissue to the endotoxin.[19-22] In some animal species, at least, the undesirable effects of an initial dose of endotoxin could be prevented by the administration of an adrenocorticotropic hormone or by adrenal corticosteroids, and the toxic manifestations of hypersensitivity to brucella endotoxin in the human being could also be subdued by the steroids.[23-24] Patients having acute brucellosis exhibit a remarkable sense of well-being shortly following steroid therapy.

Certain similarities between the physiologic changes produced by endotoxin and by serum anaphylaxis were recognized by Anderson and Rosenau[25] as early as 1909, only 7 years after the original description of anaphylaxis by Portier and Richet.[26] They predicted at that time that further studies of anaphylaxis would offer an explanation for the poisonous nature of endotoxin. However, the fundamental features of this relationship have received only sporadic attention. Bronfenbrenner[27] probed this relationship for many years and offered the provocative explanation that a fundamentally common chain of events is set off in anaphylactoid and anaphylactic activity. Nevertheless, the nature of the initial injury which triggers this chain of events is not understood. The present status of the pertinent theories has been reviewed by Burdon.[28] Since more information is needed to clarify any basic relationship that may exist between these reactions, we desire to compare our results of investigations on the response of the dog to endotoxin with the documented information on anaphylaxis in the dog.

METHODS

Preparation and Activity of Endotoxin.—Endotoxins from different species of gram-negative microorganisms have similar physiologic effects. In the present experiments, endotoxins from *Brucella melitensis, Escherichia coli, Salmonella typhosa,* and *serratia marcescens* were used.* When differences in physiologic potency of the various lots were adjusted, each of these endotoxins produced the characteristic effects in dogs which were indistinguishable from one another.

In the initial experiments to be described, *E. coli* or *Br. melitensis* endotoxin of the Boivin type was employed.[1] On the basis of exploratory experiments, a single injection of 5 mg. of *E. coli* endotoxin or 10 mg. of *Br. melitensis* endotoxin per kilogram body weight was used since these amounts regularly produced shock when injected intravenously in the dog.

Because of the large and costly amounts of endotoxin that were needed to produce shock in the dog, the response to less purified preparations was subsequently explored. A preparation of dried bacterial bodies was finally selected, which was made from *E. coli* cells grown on synthetic protein-free media according to a modification of a technique described by Braude and associates.[29] The organisms were harvested in a Sharples centrifuge and washed with alcohol and acetone. The bacterial bodies were dried and then suspended in saline to give a concentration of 50 mg. per ml. A response which was indistinguishable from that produced by the highly purified endotoxins was regularly produced in the dog following the injection of 0.3 ml. per kilogram of this preparation.

The possibility that the anaphylaxis-like reactions to endotoxin were the result of protein contaminants in the endotoxins, derived from the bacterial media, was excluded by the use of synthetic, protein-free media in the preparation of both purified and "crude" *E. coli* endotoxin. Moreover, the highly purified fractions from *S. marcescens* and *S. typhosa* were essentially protein-free.

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*Endotoxin from *S. typhosa* was supplied by Dr. Dennis Watson of the University of Minnesota and from *S. marcescens* by Dr. M. J. Shear, National Cancer Institute.

RESULTS

Clinical Manifestations—The initial observations were made with purified endotoxins from *E coli* or *Br melitensis*, which were injected intravenously into healthy adult dogs [11 12 16] Puppies were avoided because shock was not uniformly produced in the immature animal Shortly after the injection was completed, the dog had a forceful, well formed bowel movement Two to three minutes after injection, pruritus, hyperpnea, agitation, vomiting, and diarrhea were manifested The animal began to shiver markedly and the blood pressure fell progressively The dog became apathetic, resting quietly in a corner but frequently he would walk in ataxic fashion for short distances, dragging his hind legs as if paralyzed Over the first hour, gagging and tenesmus were prominent and this was subsequently followed by profuse bloody vomitus and bloody diarrhea A gradual rise in rectal temperature from approximately 39° to 42° C usually occurred during the first 3 hours Between 3 to 5 hours, the animal usually lapsed into coma, and death occurred between 12 to 24 hours after the injection of endotoxin

In their earliest writings, Portier and Richet[26] noted practically identical clinical events in sensitized dogs after the second or precipitating injection of antigen The prominence of gastrointestinal manifestations with bloody vomitus and diarrhea and the profound changes in circulation leading to shock were subsequently confirmed by many other students of anaphylaxis A minor point of difference between anaphylaxis and the reaction due to endotoxin is the elevation in body temperature which usually follows endotoxin but not serum anaphylaxis [30 31]

Pathologic Morphology—Following death of the animal from endotoxin shock the most prominent morphologic changes are found in the gastrointestinal system Severe congestion of the liver, submucosal bleeding, and epithelial slough of the surface of large portions of the small intestine, and marked edema of the gall bladder were quite uniformly present Necrosis of liver cells was noted in some animals which survived for longer than 12 hours

Essentially the same morphologic alterations have been observed in the gastrointestinal tract of dogs following anaphylactic shock [31 33] The findings in both states are probably to a large extent the result of severe engorgement and vascular stasis occurring in the portal venous system

Hemodynamic Effects—In further studies in dogs anesthesized with pentobarbital and given endotoxin, arterial and venous pressures were measured [17 18] The arterial pressure began to fall precipitously less than one minute after injection of the endotoxin was completed The portal vein pressure increased up to 4 times within the first 5 minutes but returned to near its former value after 10 to 20 minutes There was partial recovery of the arterial pressure during the first hour but subsequently the blood pressure again fell to lower values from which it did not ordinarily recover (Fig 1) The immediate fall in arterial pressure and the marked rise in portal vein pressure were traced to the accumulation of large amounts of blood in the liver and intestine This was presumably caused by transient obstruction to venous outflow from the liver The removal of large amounts of blood from active circulation because of this pooling in the portal system resulted in a critical reduction of the quantity of blood returned to the heart. A marked fall in cardiac output occurred and this was subsequently shown to be the cause of the initial fall in blood pressure.

When the liver was excluded from the circulation by hepatectomy, by evisceration with ligation of the portal vein and hepatic artery, or by portal-cava anastomosis with hepatic artery ligation, no immediate fall in blood pressure followed the injection of endotoxin.[16] These experiments clearly demonstrated that the presence of the liver is essential for this initial collapse in blood pressure.

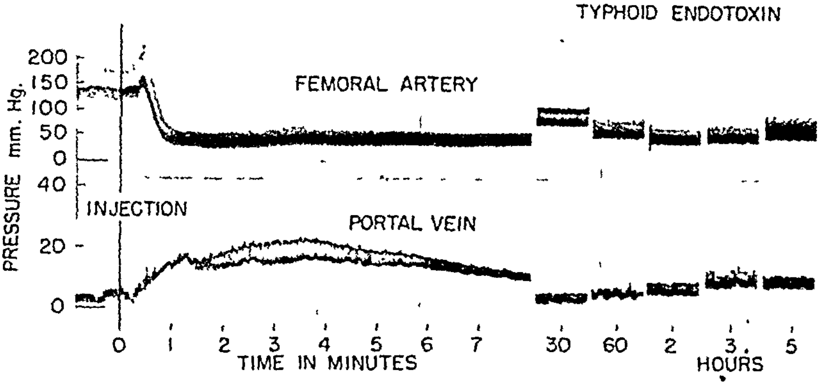

Fig. 1.—Arterial and portal vein pressure curves following administration of *S. typhosa* endotoxin to the dog. The pressures were determined with strain gauge manometer systems with appropriate damping and a polyviso recorder.

In anaphylactic shock a very similar fall in arterial pressure and elevation in portal vein pressure occurs within the first minute following injection of the antigen to which the animal has been sensitized.[31-36] This fall in arterial pressure has likewise been traced to pooling in the portal system and inadequate cardiac filling.[35-37] Manwaring[23] clearly demonstrated that exclusion of the liver from the circulation prevented anaphylaxis, and the parallel situation with endotoxin further emphasizes the fundamental similarity in hemodynamic effects in the two reactions.

Relationship of Shock to Quantitative Changes in Blood Cells, Platelets, and Blood Sugar.—The injection of endotoxin was followed by a rapid and profound fall in the number of white blood cells. A progressive reduction in the percentage of neutrophils with a relative increase in lymphocytes occurred during the first 90 minutes but the maximal fall in total leukocytes had already occurred during the first 15 minutes (Table I). A corresponding and also very marked decline in the number of platelets took place during the first 5 to 15 minutes (Table II). There was a very close relationship between the abrupt fall in total leukocytes, the decrease in platelets, the drop in arterial pressure, and the transient elevation of the portal vein pressure (Fig. 2). As the leukocyte and platelet counts gradually increased during the 3 hours that followed, a large number of immature red blood cells appeared in the peripheral circulation (Table II).

TABLE I. QUANTITATIVE CHANGES IN TOTAL LEUKOCYTES, NEUTROPHILS, AND LYMPHOCYTES AT VARIOUS PERIODS AFTER INJECTION OF E. COLI OR BR. MELITENSIS ENDOTOXIN INTO DOGS ANESTHETIZED WITH PENTOBARBITAL*

	CONTROL		TIME IN MINUTES AFTER INJECTION OF ENDOTOXIN					
	BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	90	180
Total leukocytes cells/mm.³	15,180 (5)	14,188 (4)	1,525 (2)	1,710 (5)	1,925 (4)	2,420 (5)	2,520 (5)	4,900 (3)
Neutrophils per cent	80.0 (5)	80.1 (4)	70.1 (2)	62.4 (5)	66.1 (4)	62.2 (5)	52.2 (5)	73.1 (3)
Lymphocytes per cent	18.1 (5)	17.1 (4)	28.1 (2)	35 (5)	30.3 (4)	35.1 (5)	43.4 (5)	24.2 (3)

*Blood samples were obtained from the femoral vein at the indicated times and the counts were done in duplicate. The mean values and the number of dogs (indicated by parentheses) on which each value is based are shown.

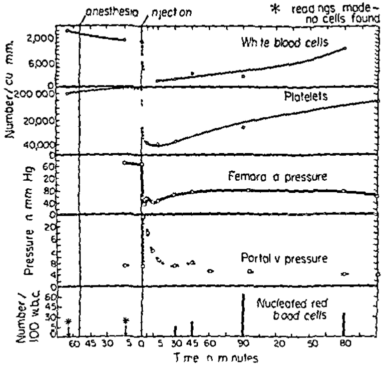

Fig. 2.—Representative hematologic changes and the related arterial and portal vein pressure responses before and following injection of crude *E. coli* endotoxin in the dog.

There was surprisingly little change in the venous hematocrit during the initial 30 minutes. This was in harmony with our earlier observation that pooling of blood in distended veins and not leakage of plasma accounted for the initial hemodynamic disturbance produced by endotoxin.[15] A slight elevation in the concentration of blood glucose during the first 15 minutes was followed by a steep fall during the ensuing 3 hours (Table II and Fig. 3).

The effects of endotoxin on blood cells, platelets, and blood sugar have been well described by other investigators.[38-41] The purpose of including these values in the present series of experiments was to determine the time relationship between these changes and the sudden fall in blood pressure. It will be noted that leukopenia and thrombocytopenia are closely associated with the sudden appearance of shock.

The close similarity between these hematologic changes and those that follow anaphylactic shock in the dog are noteworthy. Biedl and Kraus[34] first described leukopenia in anaphylactic shock and Webb[12] noted that the abrupt fall in leukocytes occurred within 2 minutes following injection of the shocking

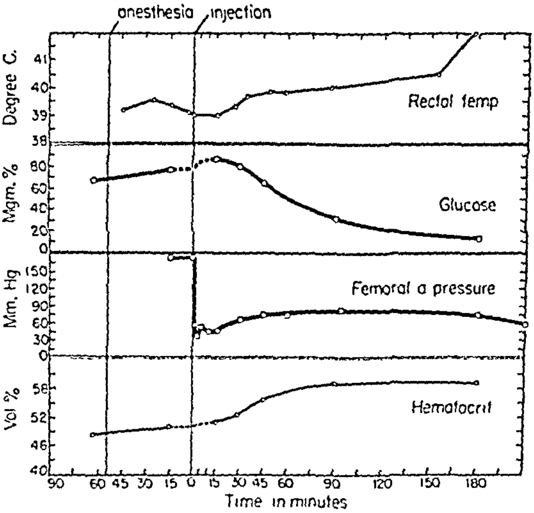

Fig. 3.—Changes in the hematocrit, concentration of blood glucose and rectal temperature, and their relationship to the fall in arterial pressure produced by crude E. coli endotoxin.

TABLE II. QUANTITATIVE CHANGES IN THE PLATELET COUNT, NUMBER OF NUCLEATED RED BLOOD CELLS, HEMATOCRIT, AND BLOOD GLUCOSE CONCENTRATION AT VARIOUS PERIODS AFTER INJECTION OF E. COLI OR BR. MELITENSIS ENDOTOXIN INTO DOGS ANESTHETIZED WITH PENTOBARBITAL*

	CONTROL		TIME IN MINUTES AFTER INJECTION OF ENDOTOXIN							
	BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	90	120	180	210
Platelet Count	217,000	242,800	55,300	40,500	62,200	86,700	96,100	75,000	96,250	177,000
No./mm.^{3}	(5)	(6)	(5)	(6)	(5)	(5)	(4)	(2)	(2)	(1)
Nucleated red blood cells	*0*	*0*	*1*	*8*	*14*	*35.4*	*53.5*		*30*	
No./100 leukocytes	(5)	(4)	(2)	(5)	(4)	(5)	(5)		(3)	
Hematocrit	51.4	46.8	53.1	50.9	50	51.7	53.6		55.3	
Vol. per cent	(5)	(4)	(2)	(5)	(4)	(5)	(5)		(3)	
Blood Glucose	69.4	81.1	80	90	81	80.2	76.4		48.1	
Mg. per cent	(5)	(5)	(1)	(5)	(4)	(5)	(5)		(3)	

*Blood samples were obtained from the femoral vein and determinations were done in duplicate. The mean values and the number of dogs (indicated by parentheses) on which each value is based are shown.

dose of antigen. However, recovery of the leukocyte count and the development of leukocytosis apparently proceed somewhat more rapidly after anaphylaxis than after endotoxin.[42, 43] The rapid development of thrombocytopenia after endotoxin is also a constant finding in anaphylactic shock.[44]

Blood Coagulation.—The coagulability of blood was measured before and at intervals after the injection of endotoxin using a modification of the Lee White method.[45] No significant change in the coagulation time was detected (Table III). Franke[46] had also noted no consistent change in clotting time in dogs after the injection of large doses of endotoxin derived from *S. marcescens*.

TABLE III. COAGULATION TIMES (MODIFIED LEE WHITE METHOD) AT VARIOUS PERIODS AFTER INJECTION OF ENDOTOXIN INTO DOGS

NUMBER AND SEX	WT (KG)	ENDOTOXIN	COAGULATION TIME IN MINUTES								
			CONTROL		TIME (MIN.) AFTER INJECTION OF ENDOTOXIN						
			BEFORE ANESTHESIA	AFTER ANESTHESIA	5	15	30	45	60	90	180
1 M	25.4	*E. coli* (crude)	9.0	8.5		8.0	9.0		6.0		7.5
2 M	15.0	*E. coli* (crude)	6.5	13.0		10.0	12.5	10.5		8.5	9.0
3 M	23.0	*Br. melitensis* (*purified*)	9.0	8.5		15.5	14.0	19.5		15.5	11.0
4 F	12.0	*E. coli* (*purified*)	7.0		8.5	8.5	6.5	8.0		9.5	
5 M	23.5	*E. coli* (crude)	6.0	6.5		6.0	6.5	9.5		8.0	6.0
Mean			7.4 (5)	9.1 (4)	8.5 (1)	9.6 (5)	9.7 (5)	11.9 (4)	6.0 (1)	10.4 (4)	8.4 (4)

The prolongation of coagulation time due to liberation of heparin is a consistent and well recognized feature of anaphylactic shock.[34, 47, 48] The lack of evidence that heparin is liberated following endotoxin may constitute a significant difference between anaphylactic shock and the shock produced by endotoxin.

Role of the Central Nervous System.—The characteristic hemodynamic and pathologic changes that follow the injection of endotoxin were not prevented in dogs after cervical chordotomy, with and without vagotomy.[11] A prompt fall in blood pressure and elevation of the portal vein pressure also occurred in decapitated animals. Using a modification of the cross circulation method of Penner and Klein,[8] which has been presumed to separate the cephalic and body circulation, shock and the classic morphologic changes could not be related to primary activity of endotoxin in the cephalic circulation. These experiments did not support the widely accepted thesis that shock results primarily from the direct effect of endotoxin on the brain.[8]

The earliest workers studying anaphylactic shock also postulated that shock was due to profound chemical effects on the central nervous system. When in 1909, Anderson and Rosenau[25] compared the manifestations of anaphylaxis and endotoxin, they proposed that both were the result of overwhelming toxic effects on the central nervous system. In refutation of these arguments, Pearce and Eisenbrey[49] demonstrated that decapitation and mechanical destruction of the spinal cord of the dog did not protect against anaphylactic shock. Anesthesia and sedation may mask the systemic symptoms of anaphylactic shock but the essential characteristics of the reaction are not modified.[34] Our observations in unanesthetized animals and in dogs following pentobarbital anesthesia also demonstrated that vomiting, diarrhea, and minor clinical signs were prevented, but the profound hemodynamic and morphologic alterations were unchanged by anesthesia. The information at hand indicates that the initial reactions produced by anaphylaxis or by endotoxin in the dog are not primarily the result of a direct action on the central nervous system.[11, 31]

Histamine Liberation.[*]—Present knowledge of the role played by histamine in anaphylactic shock is incomplete. However, following the studies of Dragstedt and his group,[50-52] liberation of histamine was recognized as a constant feature of anaphylactic shock in the dog. It was, therefore, of considerable interest to determine whether histamine is also released following the injection of endotoxin in dogs.

Method of Assay.—Bio-assay for histamine was performed with a muscle chamber using guinea pig ileum.[53] A freshly removed segment of intestine, approximately 3 cm. in length, was suspended in a 5 ml. chamber filled with Ringer-Tyrode solution. Oxygen was continuously bubbled into the chamber and the system was maintained in a 37° C. water bath. The lower end of the gut was secured by means of a hook to the bottom of the chamber and the upper end was fastened to the free end of a cantilever beam. The initial tension was adjusted to between 0.5 to 1.0 Gm. and subsequent changes in length of the gut were detected by means of a pair of strain gauges cemented to the beam. Recording of the deflection was made on a Sanborn recording system.

With alternate injections of standard quantities of histamine and 0.3 ml. of heparinized plasma, it was possible to determine the quantity of ''histamine-like'' substance in plasma. The chamber was washed two times with prewarmed Ringer-Tyrode solution between each determination.

TABLE IV. HISTAMINE-LIKE SUBSTANCE DETECTED IN PLASMA OBTAINED FROM THE SUPRADIAPHRAGMATIC INFERIOR VENA CAVA AT INTERVALS AFTER THE INJECTION OF ENDOTOXIN

DOG NO.	WT. (KG.)	SEX	ENDOTOXIN	CONTROL	MICROGRAMS PER ML. PLASMA (MINUTES)									
					1	2	3	4	5	10	15	20	30	60
1	10.2	M	*Br. melitensis* (purified)	0	0.22	0.13	0.04	0	0	0	0			
2	23.0	M	*E. coli* (cells)	0	0.13	0.04	0	0	0	0	0			
3	9.8	M	*E. coli* (cells)	0	0.03	0.09	0.03	0	0	0	0	0		
4	17.8	F	*E. coli* (cells)	0	0.64	0.39	0.36	0.13	0.08	0	0		0	0

In initial trials, samples of heparinized plasma from the femoral vein of dogs were analyzed before and at intervals after injection of endotoxin. No increase of ''histamine-like'' substance could be detected in these samples from the dog in a state of shock. When samples were collected through a cardiac catheter, which had been carefully advanced into the superdiaphragmatic inferior vena cava to a site near the inflow of the hepatic veins, ''histamine-like'' substance was readily detected. The maximum response occurred within one to two minutes after injection of endotoxin and coincided with the period of precipitate fall in the arterial pressure. Between the third and fifth minute, the material had again disappeared from the plasma (Table IV and Fig. 4).

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[*] We are indebted to Mr. R. J. Stish of the Department of Physiology who collaborated in these studies.

A sharp rise and rapid fall of blood histamine was also noted by Code[51] in his studies of anaphylaxis in the dog. The explosive liberation of histamine was likewise shown to be closely related to the onset of hypotension, elevation of the portal vein pressure, and pooling in the viscera. The present findings reveal a significant point of similarity between anaphylactic shock and the reaction to endotoxin.*

Effect of Adrenocortical Hormones.—The therapeutic value of adrenocortical hormones in allergic diseases is well recognized, but reports on the effectiveness of ACTH and cortisone in preventing anaphylactic shock in animals are contradictory.[52, 53] However, it would appear that at least partial protection can be demonstrated if the intensity of the anaphylactic reaction is suitably controlled.[53] We have observed that these hormones clearly protect against the lethal effects of endotoxin in the mouse, rat, and rabbit.[20-28] Ebert and associates[10] could not prevent shock in the dog with meningococcus toxin by pretreating animals with cortisone. In view of the important theoretical and therapeutic implications derived from a knowledge of the effects of corticosteroids on shock produced by endotoxin, this problem was re-explored.

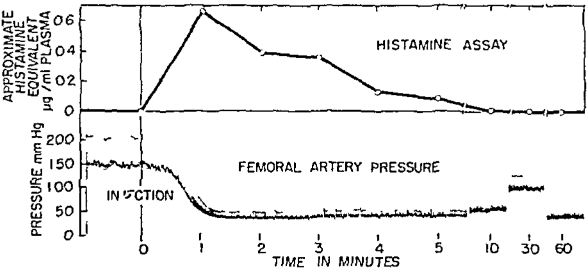

Fig. 4.—Relationship of the amount of histamine-like substance in inferior vena cava blood of the dog to the arterial pressure following injection of crude *E. coli* endotoxin.

Method of Study.—One of a pair of dogs was pretreated with large doses of hydrocortisone hemisuccinate† or prednisolone 21 hemisuccinate† which were injected intravenously 5 to 60 minutes before the endotoxin. Observations on the treated and an untreated control dog were made simultaneously and the experimental animals were so chosen that weight,

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*Recent investigations indicate that serotonin as well as histamine is liberated into the blood during anaphylaxis in the rabbit (see T. P. Waalkes, H. Weissbach, J. Bozicevich, and S. Undenfriend: J. Clin. Invest. 36: 1115, 1957).

†Supplied through the courtesy of Dr. C. J. O'Donovan, The Upjohn Company, Kalamaz
Mich.

Table V. Effect of Pretreatment With Adrenal Steroids on the Survival Time of 12 Pairs of Dogs After Injection of Endotoxin

	EXPERIMENTAL ANIMAL						CONTROL ANIMAL			
NO.	WT. (KG.)	SEX	THERAPY	TIME PRECEDING ENDOTOXIN (MINUTES)	OTHER THERAPY	SURVIVAL TIME (HOURS)	WT. (KG.)	SEX	SURVIVAL TIME (HOURS)	COMMENT
1	14.5	F	100 mg. Fh 100 mg. Fh (I.M.)	7	None	15½	10.0	F	2½	Anesthetized
2	12.5	F	100 mg. Fh 25 mg. Fh (I.M.)	8	50 mg. E at 2½ hr.	Survived	13.5	F	Survived	Anesthetized
3	11.0	F	100 mg. Fh	10	None	0	11.0	F	4½	Anesthetized; treated animal died 5 min. after injection of endotoxin
4	7.0	F	100 mg. Fh	10	None	Survived	7.0	F	18	Anesthetized
5	10.0	M	100 mg. Fh	13	None	12	10.0	M	6	Anesthetized
6	8.0	F	100 mg. Fh 50 mg. E	5	50 mg. Fh at 1 and 2 hr.	22	11.0	F	10½	Anesthetized
7	7.0	M	100 mg. Fh 50 mg. E	9	50 mg. Fh at 2 hr.	2	9.5	M	6	Anesthetized
8	11.5	M	100 mg. Fh 100 mg. E	15	50 mg. Fh at 1 and 2 hr.	10	12.0	M	5	
9	11.0	F	100 mg. Fh 50 mg. E	18	50 mg. Fh at 1 and 2 hr. 50 mg. E at 4 hr.	Survived	12.0	F	5½	
10	13.0	M	100 mg. Fh 50 mg. E	23	50 mg. Fh at 1 and 2 hr. 50 mg. E at 4 hr.	12	10.5	M	12	
11	8.2	F	35 mg. ΔFh	60	None	7½	7.8	F	5	
12	6.4	M	35 mg. ΔFh	60	None	30	10.8	M	8½	

Key to abbreviations: I.M. = intramuscular injection; Fh = hydrocortisone hemisuccinate; ΔFh = prednisolone hemisuccinate; E = cortisone acetate.

sex, and age of the treated and control dog were closely matched. Twelve treated and 12 simultaneous control animals were studied. In some experiments, supplementary doses of hydrocortisone hemisuccinate or cortisone acetate were given by the intramuscular route shortly before and at intervals after the administration of endotoxin (Table V). The same lot of "crude" *E. coli* endotoxin in a dose of 0.2 to 0.3 ml. per kilogram was used for all experiments. Seven pairs of dogs were anesthetized with pentobarbital and femoral artery pressure was recorded for 3 hours after injection of the endotoxin. In the remaining 5 experiments the animals were not anesthetized so that any clinical differences could be observed. Rectal temperatures were studied in 2 pairs.

Characteristic hemodynamic and clinical responses were not clearly altered by pretreatment with the corticosteroids. Three of the treated animals but only one control animal survived (Table V). The average survival of the 11 control animals was only 7.5 hours. These data suggest that corticosteroids provided protection under the circumstances of these experiments but the differences are not decisive. Further studies with particular attention to the dose of endotoxin may lead to a clearer definition of the possible protection afforded by these agents in the dog.

DISCUSSION

The remarkable similarity of anaphylactic shock and shock that follows the injection of endotoxin suggests that a common pathway is involved in both reactions. A single injection of peptone, trypsin, hydatid cyst fluid, snake venom, the extract of *Ascaris lumbricoides*, polyvinylpyrrolidine (PVP), acacia, and fat emulsions also produce reactions which, though not identical, simulate anaphylaxis.[39, 58, 64] Seventy-one different substances, including organic colloids such as starch, glycogen, and pancreatin, have produced the anaphylactic type of reaction, and also included are several inorganic chemicals such as arsenous sulfide and colloidal iron.[58]

"Anaphylactoid shock" has been a descriptive term that is widely used in indicating the close resemblance of the reaction to anaphylactic shock, although the basic mechanism involved in the anaphylactoid reaction is not clearly understood. Recent investigation has suggested that these reactions may be closely related to the molecular size of particles injected.[61] This would, in part, explain the diversity of chemically unrelated substances that produce this reaction. Peptones, carbohydrate polymers, fat emulsions, and colloidal solutions of certain inorganic materials are each composed of macromolecules of intermediate size. Purified endotoxins are also made up of macromolecules in the 100,000 to 1,000,000 range.[65]

The fundamental question of how macromolecules "trigger" anaphylaxis and anaphylactoid reactions in the dog still remains unexplained. The conclusion that the reactions are very closely related to changes in the liver and that they do not occur in the absence of the liver is well documented. Thomas and Essex[66, 67] have demonstrated that occlusive spasm of the intrahepatic vasculature accompanies anaphylactic and anaphylactoid shock and this conclusion is supported by studies from this laboratory.[12, 15, 16] Spasm of small hepatic veins explains the elevation of portal venous pressure, severe engorgement of the liver and intestine and marked edema of the gall bladder. Shock results from the pooling of blood behind the small tributaries of hepatic veins with resulting loss of large quantities of blood from the active circulation. This effect is most important in producing the profound hypotension that so rapidly follows injection of the shocking agent. The role of histamine in this sequence of events has been much debated but present evidence suggests that anaphylaxis and anaphylactoid shock are not the result of its action.[28, 18, 68]

In contrasting anaphylactic shock and the reaction to endotoxin in the dog, failure to obtain significant prolongation of the coagulation time with endotoxin has already been cited as an important point of difference. In addition, certain delayed effects of endotoxin may not be directly related to its anaphylactoid activity. One such delayed effect has been noted after hepatectomy. Whereas the initial response of shock following injection of endotoxin is prevented by preliminary hepatectomy, the lethal effect is augmented.[16] This is in contrast to the absence of any injurious effect when attempts are made to provoke anaphylaxis in a sensitized dog after the liver has been removed. Braude and associates[69] have shown that circulating endotoxin is, in fact, removed by the liver so that the absence of the liver may cause intensification of a delayed toxic effect which is apparently not related to its anaphylactoid activity. Studies are now in progress to define the nature and importance of this delayed effect more fully. These differences, however, do not minimize the essential similarity of the reactions produced by endotoxin, anaphylaxis, and anaphylactoid shock.

The occurrence of sudden shock in human patients with bacteremia caused by gram-negative microorganisms is probably related to the liberation of endotoxin from the bacteria. The clinical manifestations in severe cases may resemble anaphylaxis with sudden prostration, profound hypotension, bloody vomiting and bloody diarrhea, and a rapidly fatal course.[3, 5] Severe congestion of the liver, necrosis of liver cells, and submucosal hemorrhage, not unlike that observed in the experimental animals, are commonly found at autopsy.[70]

These studies admittedly leave the fundamental questions concerning pathogenesis unanswered. However, the concept that bacteremic shock is an anaphylactoid response permits a larger body of experimental and clinical knowledge to bear on this problem. Indeed, studies to date emphasize the need for further knowledge of the nature of the "trigger" which initiates the sequence of events in anaphylaxis or anaphylactoid shock.

SUMMARY AND CONCLUSIONS

When endotoxins from gram-negative microorganisms were injected intravenously into dogs, the animals became severely ill. The following series of events was observed: a precipitous fall in blood pressure, marked hyperpnea, excitement and gagging, apathy, and later coma. Massive evacuation of the bowels and vomiting occurred soon after the injection and this frequently led to profuse bloody vomitus and tenesmus with bloody diarrhea. Death followed within 3 to 18 hours and autopsy showed severe liver congestion, submucosal intestinal bleeding, and gall bladder edema.

The onset of shock was accompanied by a rise in portal vein pressure and the cause of shock was traced to massive pooling of blood in the portal system resulting in a greatly diminished venous return. An immediate marked fall in leukocytes and platelets also followed the injection of endotoxin The blood sugar was slightly elevated but subsequently fell to low levels after the second hour Chordotomy, vagotomy, and decapitation did not alter the essential hemodynamic response and this indicated that shock was not primarily due to intoxication of the central nervous system Histamine like substance appeared in the blood of the inferior vena cava immediately after shock was produced Pretreatment with adrenocortical hormones provided only questionable protection against the reaction These studies demonstrated that shock produced by a single injection of endotoxin was, in each of these respects, simulated to those documented in the case of anaphylactic shock in the dog

Two important points of difference were noted between shock caused by endotoxin and that due to anaphylaxis No change in blood coagulability occurred after endotoxin, and this was in contrast to the liberation of heparin and prolongation of clotting time which characteristically occurs during anaphylaxis Moreover, endotoxins appear to have an additional delayed toxic effect that leads to rapid death after hepatectomy

The reaction to endotoxin is thought to be another example of the anaphylactoid type of reaction Its close relationship to shock produced by a diversity of unrelated agents, including peptones, carbohydrate polymers, parasitic extracts, and snake venoms and the shock in patients with bacteremia which produce a similar response, have been discussed

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TRANSMISSION OF THE EXPERIMENTAL COMMON COLD IN VOLUNTEERS

II. The Effect of Certain Host Factors Upon Susceptibility

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The reasons why some persons have more colds than others are not known. The age of the subject[1-4] and the frequency and intimacy of exposure to persons with a common cold[5] have been shown to be important factors. Women acquire "natural" colds more frequently than men[1, 4, 6, 7] and more frequently develop the common cold after experimental inoculation.[8] Other unknown factors appear to influence the development of the common cold, however, since certain persons remain more susceptible to colds from year to year[2, 9, 10] and some persons are more susceptible to repeated challenges with infectious secretions than others.[8]

In the course of observations of the development of the common cold in adult volunteers following nasal instillation of infectious secretions, we attempted to study any additional factors which might be related to susceptibility to the common cold. We are presenting herewith our findings with regard to allergy, the presence or absence of tonsils and adenoids, the smoking of tobacco, and menstruation. Observations on the effect of chilling, as such, upon the development of the experimentally transmitted common cold are being reported separately.[11]

MATERIALS AND METHODS

Secretions obtained from patients with typical common colds were filtered through an ultrafine sintered glass filter. After bacteria were shown to be absent by culture and there was no demonstrable effect upon embryonated eggs, suckling mice, adult mice, or on HeLa cells in tissue culture, the secretions were diluted 1:10 and stored in a 5 per cent solution of hemoglobin, obtained from group O cells buffered to a pH of 7.4 at -70° C. Immediately preceding instillation the solution was again diluted 1:10 with 0.15 M phosphate buffered saline solution, pH 7.4. The control for these secretions was a solution of hemoglobin obtained from group O cells, buffered in the same way. Inoculations were made by instilling 0.2 ml. of the solution into each nostril.

After these laboratory studies were performed on each secretion, it was instilled into the nares of 6 to 10 human volunteers to determine whether it would cause symptoms of the common cold. All volunteers were healthy men or women between the ages of 18 and 35 years.

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Special rooms or chambers were used to control the temperature and humidity of the environment Men remained for 2 to 4 hours in the specified environment, dressed in shorts and socks, women in shorts, socks, and halters or brassieres A chilling temperature of 60° F with an 80 per cent relative humidity for 4 hours and a comfortable temperature of 80° F with a 30 per cent relative humidity were selected for the experiments The infectious secretions or control solutions were instilled intranasally immediately prior to chilling or at the end of the period of chilling

For experiments at lower temperatures (10° F and 80 per cent relative humidity for 2 hours) the subjects were dressed in street clothes plus overcoat, hat, and gloves At the end of the period in the special temperature rooms, the intranasal instillations were made and the subjects were dismissed They reported for examinations on the second, third, fourth, and sixth days after the experiment Initial examinations of the nose and throat were made and a history of respiratory symptoms was obtained at the beginning and end of each experiment and on each of the subsequent visits These examinations, and the subsequent decision as to whether or not a cold was present, were made by members of the research team who were unaware of the experimental group into which the patient had been placed

The criteria for identifying the development of a cold in a volunteer and the reasons for their adoption will be given in detail in another publication[19] In brief, in order to be classified as having developed a cold in connection with an experiment, the subject had to meet 2 of the following 3 criteria

1 A score of 14 or more points in 6 days (over the base line symptoms taken on the day of the experiment) based on the following symptoms recorded daily headache, sneezing, chilliness, nasal discharge, nasal obstruction, sore throat, coughing, and malaise

2 The impression of the subject that a cold had developed following challenge

3 An increase in nasal discharge on 3 of the 6 days after challenge (or severe nasal discharge on 2 days)

RESULTS

History of Allergy—In Table I is shown the frequency with which colds developed in allergic and nonallergic persons A history of allergy was given by 143 of the subjects who were challenged with one of 5 infectious secretions obtained from patients with the common cold Colds developed in 64 or 45 per cent of these subjects These results are to be compared with 218 (31 per cent) colds that developed in 693 persons who also received one of the same 5 infectious secretions but gave no history of allergy

Among subjects who gave a history of allergic symptoms following the inhalation of certain substances, colds developed more frequently (48 per cent) than among subjects whose allergic symptoms followed the injection or ingestion of particular substances (40 per cent) The statistical possibility that the differences between these 2 groups and the nonallergic group occurred by chance was less than 1 in 100

The differences among the male and the female subjects showed the same trend as in the entire group However, the number of observations in the female groups were insufficient to give statistical significance to the differences observed

Lines 1 to 4 in Table I represent subjects who received no infectious secretions and whose upper respiratory infections, therefore, represented spontaneous "colds" Spontaneous infections were more frequent in subjects with

TABLE I. FREQUENCY OF COLDS IN RELATION TO A HISTORY OF ALLERGY

LINE NO.	RECEIVED INFECTIOUS SECRETION	TYPE OF ALLERGY	MEN			WOMEN			BOTH SEXES		
			NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD	
				NO.	PER CENT		NO.	PER CENT		NO.	PER CENT
1	No	None	125	13	10	126	13	10	251	26	10
2		Inj.-Ing.*	10	1	--	14	2	--	24	3	13
3		Inhalant	18	2	--	16	3	--	34	5	15
4		All allergic	28	3	11	30	5	17	58	8	14
5	Yes	None	380	101	27	313	117	37	693	218	31
6		Inj.-Ing.*	33	12	36	22	10	46	55	22	40
7		Inhalant	69	33	48	19	9	47	88	42	48
8		All allergic	102	45	44	41	19	46	143	64	45
Lines 1 vs. 2 vs. 3			Not significant			Not significant			Not significant		
Lines 5 vs. 6 vs. 7			$X^2 = 13.07$; $P = 0.01$			$X^2 = 1.24$; $P = 0.1$			$X^2 = 10.04$; $P = 0.01$		

*Allergic reactions from substances injected or ingested.

TABLE II. FREQUENCY OF COLDS IN RELATION TO HISTORY OF SMOKING TOBACCO OR OF TONSILLECTOMY

RECEIVED INFECTIOUS SECRETION	HISTORY	MEN			WOMEN			BOTH SEXES		
		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD		NO. OF SUBJECTS	DEVELOPED COLD	
			NO.	PER CENT		NO.	PER CENT		NO.	PER CENT
No	Tonsillectomy	66	6	9	81	7	9	147	13	9
	No tonsillectomy	89	12	14	73	10	14	162	22	14
Yes	Tonsillectomy	209	66	32	154	56	36	363	122	34
	No tonsillectomy	265	68	26	199	79	40	464	147	32
No	Tobacco smoking	34	5	15	44	6	14	78	11	14
	No tobacco smoking	32	4	13	72	7	9	111	11	10
Yes	Tobacco smoking	130	47	36	119	41	34	249	88	35
	No tobacco smoking	156	43	28	172	69	40	328	112	34

inhalant allergy (15 per cent) than in those with allergy to injected or ingested substances (13 per cent) which in turn were more frequent than in subjects with no history of allergy (10 per cent) These differences, however, were not statistically significant

Presence or Absence of Tonsils and Adenoids—Subjects were questioned as to whether their tonsils and adenoids had ever been removed The answers were verified by an examination of the nose and throat The difference in the number of colds that developed in the 2 groups was negligible (Table II)

Among the subjects who received infectious secretions and whose tonsils had previously been removed, 122 of 363 (34 per cent) developed colds, whereas 147 of 464 (32 per cent) of subjects who gave no history of tonsillectomy developed colds Among the subjects who did not receive infectious secretions and whose tonsils had previously been removed, 13 of 147, or 9 per cent had symptoms of a cold, as compared with 22 of 162, or 14 per cent, of subjects who still retained their tonsils The differences between tonsillectomized and nontonsillectomized subjects were not statistically significant in either the infected or the noninfected group

Tobacco Smoking—Information regarding smoking habits was available in 328 subjects who received infectious secretions and in 111 subjects who received control solutions intranasally The subjects stated whether or not they were smokers at the time of the experiment, but in practically every instance those who were not smoking at the time of the experiment had never smoked at any time All were cigarette smokers, although some occasionally smoked a pipe also There was no significant difference in the frequency of experimental colds in the two groups (Table II)

Among the subjects who received infectious secretions, 35 per cent of the smokers and 34 per cent of the nonsmokers developed colds Among those who received control solutions, 14 per cent of the smokers and 10 per cent of the nonsmokers had symptoms of a cold The differences between the smokers and nonsmokers were not statistically significant in either group

Time in Menstrual Cycle When Experiment Was Performed—Information with regard to menstruation is available on 54 female subjects who were chilled during an experiment and 232 who were not subjected to chilling Each subject was asked the date of the onset of her last menstrual period Susceptibility appeared greater during the intermenstrual period and perhaps related to ovulation Accordingly, the data were tabulated upon the assumption that ovulation occurred at the mid point of the menstrual cycle, which was assumed to be fourteen days from the beginning of the menstrual period that immediately preceded the experiment In Figs 1 and 2, and Table III, the frequency with which colds developed in these subjects is shown in relation to the time before or after the mid point of the menstrual cycle at which the experiment was performed (i e, the infectious challenge or control solution was given)

Among the subjects who were not chilled, and who were challenged in the interval between the onset of the menstrual period and the fifth day before the mid-point of the menstrual cycle (that is, in the menstrual and immediately postmenstrual phase of the cycle) 23 per cent developed colds. When the challenge was given during the mid-portion, or the ovulatory phase, of the menstrual cycle, 37 per cent of the subjects developed colds. An infectious challenge given during the last or premenstrual part of the cycle caused the highest percentage of colds (41 per cent). These differences were not considered significant, except possibly in one instance. The probability that the difference between Lines 5 and 7 occurred by chance was less than 1 in 25.

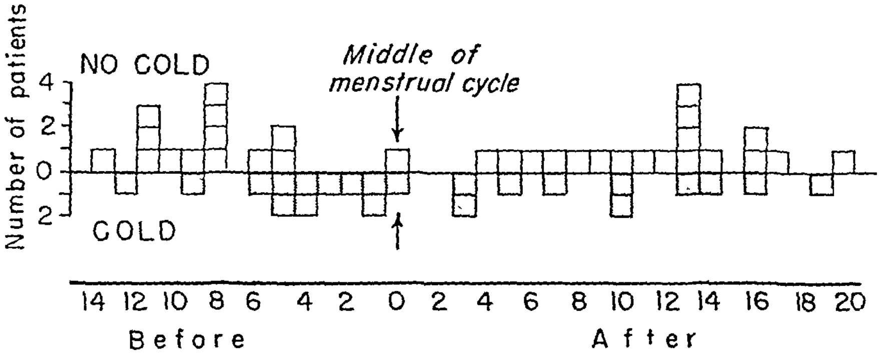

Fig. 1.—Number of experimental colds in relation to the mid-point in the menstrual cycle in subjects who were chilled.

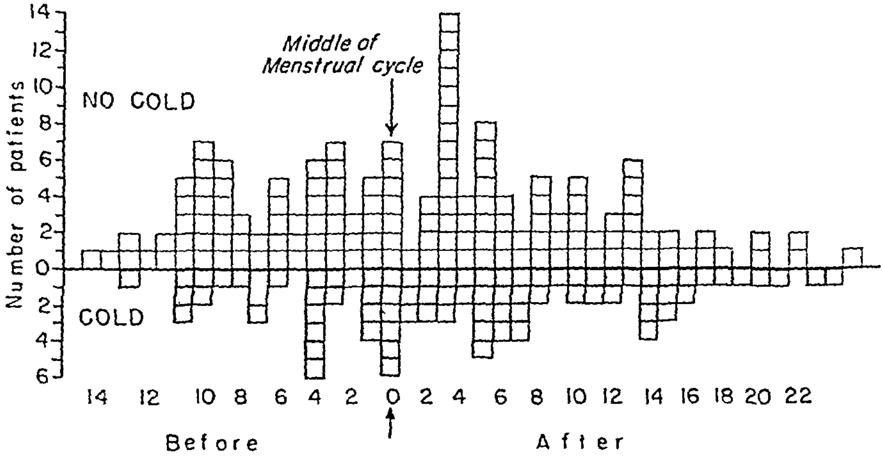

Fig. 2.—Number of experimental colds in relation to the mid-point in the menstrual cycle in subjects who were not chilled.

Among the chilled subjects, colds developed much more frequently in subjects challenged during the ovluatory phase, which was assumed to be from 4 days before to 5 days after the mid-point of the menstrual cycle (10 colds in 13 subjects) as compared with subjects challenged earlier in the menstrual cycle (5 colds in 18 subjects) or those challenged later in the menstrual cycle (7 colds in 23 subjects). The probability that these differences occurred by chance was less than 1 in 100.

When the incidence of colds among all the chilled patients was compared with that among the patients who were not chilled during the experiment the difference was not significant. When the 2 groups of subjects who were challenged during the ovulatory phase of the menstrual cycle were compared, the incidence of colds was much greater among the chilled subjects (10 colds in 13 subjects) than among subjects who were not chilled (36 colds in 97 subjects). The probability that this occurred by chance was less than 1 in 100.

TABLE III. FREQUENCY WITH WHICH EXPERIMENTAL COLDS DEVELOPED IN WOMEN. RELATIONSHIP OF MID POINT IN MENSTRUAL CYCLE TO THE DAY ON WHICH THE EXPERIMENT WAS CONDUCTED

LINE NO.	SUBJECT TO CHILLING	DAY OF EXPERIMENT (NUMBER OF DAYS BEFORE OR AFTER MID POINT OF MENSTRUAL CYCLE)	NUMBER OF SUBJECTS: CHALLENGED WITH INFECTIOUS SECRETION	DEVELOPED COLDS: NO.	DEVELOPED COLDS: PER CENT
1	Yes	5 days or more before	18	5	28
2	Yes	4 days before to 5 days after	13	10	77
3	Yes	6 days or more after	23	7	30
4	Yes	Total chilled	54	22	41
5	No	5 days or more before	52	12	23
6	No	4 days before to 5 days after	97	36	37
7	No	6 days or more after	83	34	41
8	No	Total unchilled	232	82	35

	χ^2	P		χ^2	P
Line 1 vs. line 2	7.30	0.01	Line 3 vs. line 7	0.84	N.S.
Line 2 vs. line 3	7.20	0.01	Line 4 vs. line 8	0.55	N.S.
Line 2 vs. line 6	7.47	0.01	Line 5 vs. line 6	3.05	N.S.
Line 2 vs. line 5	13.47	0.001	Line 5 vs. line 7	4.55	0.04
Line 2 vs. line 7	5.85	0.02			

DISCUSSION

Data obtained from clinical histories, epidemiologic investigations, and experimental infections[13, 14] all indicate that some individuals develop colds more frequently than others. It is extremely important to delineate the host factors that are related to susceptibility to the experimental common cold in order to find the most favorable situations under which the experimental cold may be produced and, if possible, to find the nature of the resistance to infection.

We have found that tobacco smoking and the prior removal of tonsils bear no relationship to the frequency of development of the experimental common cold. Buck[4] reported that tobacco smoking had no appreciable effect on the incidence of naturally acquired colds in a group of 45 families.

With regard to tonsillectomy, our observations are in accord with the conclusions of most observers who studied the relationship of previous tonsillectomy to the development of naturally acquired colds.[15-19] The decrease in the frequency of the common cold which follows a tonsillectomy in some children can be accounted for by one or both of the following facts. First, the tonsils are often removed because of an unusually high frequency of upper respiratory infections in the years immediately preceding the operation, whereas these infections may reach a frequency that is nearer the average after or within a year or two after the tonsillectomy. Second, since each child is older after the tonsillectomy than before, the frequency of colds is likely to be less.

The statement is often made that allergy is predisposing to colds. Siegel and his associates[20] stated that among a group of adults who reported 5 or more colds a year 19.3 per cent gave a history of asthma, hay fever, allergic rhinitis, or eczema. Among those who reported less than 3 colds a year, only 10.1 per cent gave a similar history. A family history of allergy and eosinophilia of the nasal secretions were also more frequent among the subjects who reported 5 or more colds a year. Our experiments demonstrated a slight but significant increase in susceptibility to the common cold in subjects who gave a history of allergy. It might be contended that the solutions that were instilled in the subjects' nostrils contained substances to which certain subjects were allergic. We doubt the validity of this explanation since the solutions were nasal secretions that had been rendered cell-free and for the most part bacteria-free and to which only hemoglobin obtained from group O cells had been added. Furthermore, we believe that the rigid criteria that were applied for the diagnosis of a cold would eliminate a mild allergic rhinitis, whereas the observers and the allergic subjects themselves would have diagnosed a more severe allergic rhinitis had it been present. At any rate, whatever the mechanism, it is obvious that more persons with a history of allergy have coryzal symptoms of significance after exposure to an infectious agent than persons without allergy.

The increased incidence of colds among subjects who were challenged with infectious secretion and subjected to chilling during the mid-portion of the menstrual cycle is an extremely interesting observation. We know of no information on this subject with regard to the common cold, but Weinstein and his associates[21] studied the relationship of the time during the menstrual cycle when the clinical manifestations of poliomyelitis appeared. Among 50 women, the menstrual period began in the 5 days before the first clinical manifestation of poliomyelitis in 30 per cent and in the 4 days after the onset of the disease in 48 per cent. In 14 per cent of the patients the menstrual period began from the fourteenth to the sixth day before the disease and in only 8 per cent the menstrual period started somewhere between the fifth and thirteenth day after the onset of the disease. This relationship was not considered to be the result of a change in the length of the intermenstrual interval, since none of the patients gave a history of an early or late onset of menstruation for that particular month. The authors concluded that "there may be a critical point in the menstrual cycle in respect to susceptibility to clinical poliomyelitis at an interval corresponding to the incubation period preceding the beginning of menstruation or about 12 days prior to the onset of menstrual flow."

We have recalculated these data to determine the relationship of the beginning of a 12-day incubation period to the mid-point of the menstrual cycle (assuming a 28-day menstrual cycle); as shown in Fig. 3, the presumed beginning of the incubation period fell between the 4th day before and the 5th day after the mid-point of the menstrual cycle in 39 (78 per cent) of the 50 cases This is the same period in which we found the greatest frequency of development of experimental colds.

Why subjects who are challenged and subjected to chilling around the middle of the menstrual cycle are more likely to develop a common cold than subjects who are challenged at that time without being chilled or than subjects who are challenged and chilled during another part of the menstrual cycle is a matter for speculation It has long been noted that engorgement of the nasal mucosa occurred during the premenstrual and menstrual periods and during pregnancy.[22, 23] Amberson[24] observed congestion of the bronchial mucosa around the time of the menstrual period. Mortimer and his associates[23] found that the injection of estrogens in monkeys could produce turgescence of nasal mucous membranes On the other hand, Inouye, Jackson, Dowling, and Spiesman[25] were unable to establish any correlation between the appearance of the mucous membranes of the nose at the time of challenge with an infectious secretion and the subsequent development of a common cold It is possible that estrogen or some other endocrine secretion acts upon the general metabolism or in some other way to produce resistance to the common cold

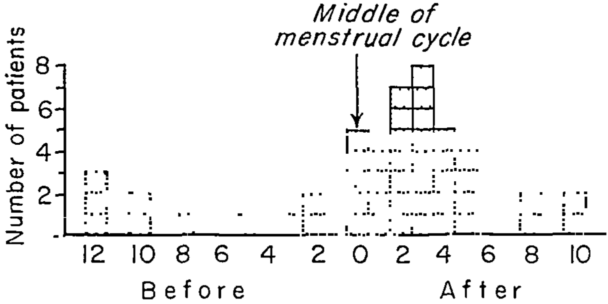

Fig. 3.—Onset of cases of poliomyelitis in relation to the mid-point in the menstrual cycle (Recalculated from Weinstein and associates[21])

SUMMARY

1. Among 143 allergic subjects between the ages of 18 and 35 years who were challenged with infectious secretions taken from patients with a common cold, 45 per cent developed colds, as compared with 31 per cent among 693 nonallergic subjects who were challenged in the same way. These differences were statistically significant.

2. Among 363 subjects whose tonsils had previously been removed, colds followed experimental challenge in 34 per cent, as compared with 32 per cent colds among 464 subjects whose tonsils were intact.

3. Among 249 persons who smoked tobacco, 35 per cent developed colds following experimental challenge, as compared with 34 per cent among nonsmokers.

4. Among 13 female volunteers who were chilled and given an infectious secretion during the middle third of the menstrual cycle, 10 (77 per cent) developed colds as compared with 28 and 30 per cent of subjects similarly treated who were challenged during the first or the last third of the menstrual cycle, respectively. The differences between the incidence of colds in chilled subjects challenged during the middle third and those chilled and challenged during the first or last thirds of the menstrual cycle or those challenged during the middle third of the menstrual cycle without being chilled, were statistically significant.

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STUDIES ON THE ROUTINE LABORATORY DIAGNOSIS OF COXSACKIE GROUP B VIRUS INFECTIONS

I. The Application of Tissue Culture Procedures

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Viruses of the Coxsackie B group have been isolated from cases of pleurodynia, aseptic meningitis, suspected or proved poliomyelitis, nondifferentiated febrile illnesses, and even apparently healthy individuals.[1] While in some of the cases an etiologic relationship could be clearly established, in others the presence of the virus might have been coincidental. In any event, it is obvious that a virus diagnostic laboratory must have convenient routine procedures for isolation and identification of these agents and for detection of antibody responses in the patients.

While the use of suckling mice led to the discovery of the Coxsackie B viruses, it does not lend itself readily to large-scale testing such as a virus diagnostic laboratory may be called upon to perform. Identification of isolated agents by serologic typing and serum neutralization tests with the patients' sera would require too large a number of animals. Crowell and Syverton[2] were the first to report on adaptation of B_1, B_3, and B_4 viruses to tissue cultures of HeLa cells but they experienced difficulties in cultivating B_2. A new member of the group, B_5, was isolated in tissue culture by Steigman.[3] Sickles and associates[4, 5] reported on the use of tissue cultures of HeLa or monkey renal cells for isolation of certain Coxsackie A and Coxsackie B viruses.

This report deals with the development of techniques, their application to routine diagnosis of sporadic cases of Coxsackie B infections, and comparisons in a few instances of the results recorded in tissue culture with those obtained in suckling mice.

MATERIALS AND METHODS

Tissue Culture Techniques.—The HeLa strain of human cancer cells was employed in these studies. Initially it was maintained as a laboratory stock culture as described by Scherer and associates,[6] and the growth medium used was composed of 40 per cent human serum, 58 per cent Hanks' balanced salt solution (BSS), and 2 per cent acetone-extracted chick embryo extract. Later the growth medium used was 20 per cent human serum and 80 per cent Basal Medium (Eagle) in Hanks' BSS.[7] Before test tube culture production, the sheets of cells in the stock culture flasks were washed with 3 changes of 10 ml. of Hanks' BSS and the cells were then suspended by trypsinization or by scraping with a rubber policeman into a medium which was composed of 10 per cent horse serum, 60 per cent maintenance solution of Scherer,[8] and 30 per cent tissue culture medium No. 199. More

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recently the medium employed has been 10 per cent horse serum and 90 per cent Basal Medium (Eagle) in Hanks' BSS The tube cultures were seeded with 0 6 ml of the cell suspension containing approximately 6×10^{4} cells After two days this growth medium in the tubes was replaced with 1 0 ml of maintenance medium containing, during earlier experiments, 10 per cent horse serum, 30 per cent No 199 and 60 per cent Earle's BSS reinforced with a small amount of casein hydrolysate* (final concentration 1 500) For later experiments 10 per cent horse serum and 90 per cent Basic Medium Eagle in Earle s BSS were employed The Earle s BSS was substituted for the Hanks BSS in the tube maintenance medium because the higher buffering capacity of the Earle's solution allowed longer incubation without fluid renewal

Viruses —

Laboratory strains The B_1 (Connecticut 5) and the B_4 (Powers) strains were obtained through the courtesy of Dr R S Huebner of the National Institutes of Health, the B_2 (Redmond) and B_3 (Nancy) strains from the American Type Culture Collection and the B_5 strain from Dr G Dalldorf in the fifth mouse passage All strains were subjected to 2 to 6 further passages in suckling mice in this laboratory prior to adaptation to tissue culture

Clinical specimens Stools were received by the virus diagnostic laboratory from patients with suspected Coxsackie or poliomyelitis infections Twenty per cent suspensions were prepared in sterile distilled water and clarified by centrifugation at 2 500 r p m for 20 minutes The supernatant fluids were treated with penicillin and streptomycin (1,000 units and 1 0 mg per milliliter, respectively) and incubated at 36° C for 60 minutes prior to inoculation into culture tubes Where mold contamination was found, mycostatin (Squibb) was employed in final concentration of 1,000 units per milliliter of stool suspension Later the stool specimen was swabbed with a cotton swab which was placed into 2 0 ml of Hanks' BSS containing the above mentioned amounts of penicillin, streptomycin, and mycostatin The swab was incubated in the Hanks' solution for 30 minutes at 37° C, excess fluid was drained, and the swab was discarded Numerous comparisons between the former and the latter method of preparation of stool specimens showed a good correlation Since the stool swab method was technically the easier and speedier one, it was finally selected as the routine method

Rectal and throat swabs These were placed into 2 0 ml of Hanks' BSS and frozen at the time of collection The fluid was expressed from the swab after thawing and the swab was discarded The preparation was lightly centrifuged (when necessary) prior to treatment with penicillin, streptomycin, and mycostatin as described above

Spinal fluids The fluids, collected aseptically and frozen until used, were not subjected to incubation with antibiotics

All specimens were inoculated into 3 culture tubes each, employing 0 2 ml volumes, or intraperitoneally into one litter of suckling mice each, using 0 03 ml per animal

Immunologic Typing of Isolated Viruses—The media from cultures showing cellular degeneration were harvested and routinely diluted to 10^{2} after it was found, on numerous titrations of such fluids, that the virus titers were approximately 10^{4} to 10^{5} The diluted fluid, 0 3 ml volume, was added to each of 4 tubes containing 0 3 ml of appropriately diluted hyperimmune sera, respectively (1) Polio Coxsackie B mixture, a pool containing poliovirus types I to III antisera and Coxsackie group B types 1 to 5 antisera, (2) Polio mixture a pool of poliovirus types I to III antisera, (3) Coxsackie B mixture, a pool of Coxsackie B types 1 to 5 antisera, and (4) Hanks' BSS solution

After one hour at room temperature, culture tubes were inoculated with 0 2 ml of one of the various sera virus mixtures and incubated at 36° C until tissue degeneration was seen in the control, usually 24 to 48 hours after inoculation After it was established whether the isolate belonged to the poliomyelitis group or Coxsackie B group of viruses, the virus type was determined, using type specific sera following the above procedure

Virus Titrations in Mice—Serial tenfold dilutions were prepared in phosphate buffered saline and one litter of 1 or 2 day suckling mice (8 mice per litter) was inoculated per dilution employed. Each animal received 0.03 ml. of the appropriate dilution by the intraperitoneal route. They were observed twice daily for a period of 2 weeks for typical paralysis and death.

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*Parenamine Winthrop Stearns Inc New York N Y

Virus Titrations in Tissue Culture.—These were performed in duplicate cultures of HeLa cells employing as inoculum 0.2 ml. of virus in serial tenfold dilutions in Hanks' BSS. Cultures were observed daily for at least 7 days.

Sera.—Hyperimmune sera were prepared in 5- to 6-week-old Syrian hamsters employing hamster-adapted Coxsackie B virus strains. For immunization, 0.2 ml. of a 20 per cent suspension of infected suckling hamster muscle was inoculated intraperitoneally. Three injections were given at 6-day intervals and the animals were bled by cardiac puncture one week after the third inoculation.

Patients' sera were prepared from bloods collected as near to the time of onset of illness as possible and 2 to 3 weeks later. These were inactivated at 56° C. for 30 minutes and stored at -20° C. until serum neutralization tests were performed.

Serum Neutralization Tests.—*Hyperimmune animal sera:* Virus and serum dilutions were prepared in Hanks' BSS (containing ⅛ the usual amount of $NaHCO_3$) and 0.3 ml. of a virus dilution was added to 0.3 ml. of a serum dilution. Following incubation of the mixtures for one hour at room temperature, 2 tissue culture tubes were inoculated with 0.2 ml. each and incubated at 36° C. Cultures were examined for 8 days before terminating the experiment. Suckling mice were inoculated with 0.03 ml. of the serum-virus mixtures, one litter per preparation, and observed for a period of 2 weeks.

Each test was accompanied by virus titrations. The viruses were initially titrated in the presence and absence of normal hamster serum. However, since no differences in titers were noted, Hanks' BSS was employed in later control titrations.

Patients' sera were diluted in twofold steps in Hanks' BSS starting at a 1:10 or 1:16 dilution, and 0.3 ml. volumes were distributed into 5 sets of tubes. Tissue culture fluids of the 5 prototype viruses, diluted to contain 100 tissue culture doses per 0.3 ml. (based on a 2-day cytopathogenic end point), were then added to the serum dilutions; sets 1 to 5, respectively. Mixtures containing 0.3 ml. of the initial serum dilution and 0.3 ml. of Hanks' BSS served as a serum control for each specimen. Incubation, inoculation, and virus titrations were carried out as outlined above. The results were recorded after the control virus titrations indicated that 100 tissue culture doses were being employed as virus challenge (48 hours).

When suckling mice were employed for the neutralization tests with patients' sera, 100 LD_{50} of mouse-adapted virus were employed, and the animals observed for a period of 2 weeks.

RESULTS

The Development of Standard Virus and Antisera.—Preliminary investigation dealt with adaptation of the 5 type B strains to growth in HeLa and with the specificity of hyperimmune sera in tissue culture neutralization tests.

1. *Adaptation of type B Coxsackie strains to growth in cultures of strain HeLa:* Passages in vitro were performed by inoculating 0.1 ml. of a dilution of infectious material (prepared in Hanks' BSS) into culture tubes containing 1.0 ml. of the maintenance fluid. On first passage, the 5 prototype strains produced definite cytopathogenic effects even in dilutions of $10^{-3.0}$ or greater (Column 2 of Table I). However, for rapid serial passages at 1- or 2-day intervals, lower dilutions were employed through the first few passages. Later passages were performed close to the limiting infective dilutions. After 6 passages in vitro the original infected mouse tissue had been diluted beyond the point where residual virus or toxicity of mouse tissue could be considered as the cause of degeneration in the cultures. These dilutions are listed in Column 5 of Table I. The differences in the final dilution factors are due to passage of

the 5 strains at varying dilutions. Furthermore, as will be shown below, the cytopathogenic changes were prevented by specific hyperimmune sera, establishing the specificity of the cellular degeneration.

Comparative titrations were performed in suckling mice and in HeLa culture tubes with both the starting seed (infected mouse tissue) and the culture fluids after 6 passages of the virus in vitro. The results are listed in Table I, Columns 1 to 4. The tissue culture titration results include the day when definite degeneration was first observed in tubes *at the titration end point*.

TABLE I. THE RESULTS OF TITRATIONS IN SUCKLING MICE AND TISSUE CULTURE (HELA) OF COXSACKIE B VIRUS STRAINS BEFORE AND AFTER PASSAGE IN VITRO

VIRUS STRAINS	ORIGINAL MOUSE VIRUS		TISSUE CULTURE PASSAGE NO. 6		DILUTION OF ORIGINAL VIRUS BY PASSAGE IN VITRO
	MOUSE LD_{50}/0.03 ML 1	TCD*/0.2 ML 2	MOUSE LD_{50}/0.03 ML 3	TCD/0.2 ML 4	5
B_1	$10^{6.5}$	$10^{5.0}$ 4.5 days†	$10^{6.5}$	$10^{7.0}$ 4 days†	10^{22}
B_2	$10^{5.5}$	$10^{4.0}$ 8 days	$10^{3.2}$	$10^{4.0}$ 5 days	10^{16}
B_3	$10^{6.0}$	$10^{5.0}$ 4.5 days	$10^{5.0}$	$10^{6.0}$ 3 days	10^{20}
B_4	$10^{6.5}$	$10^{3.0}$ 6 days	$10^{5.0}$	$10^{3.5}$ 3 days	10^{13}
B_5	$10^{6.5}$	$10^{4.5}$ 9 days	$10^{5.2}$	$10^{5.5}$ 6 days	10^{10}

*TCD = tissue culture doses

†Days of incubation when degeneration was first noted at end point dilution

The infected mouse tissues had higher titers in mice than in tissue cultures for all 5 group B types, but on passage in vitro the mouse titers decreased for all but type B_1. Simultaneously, there was an increase in the tissue culture titers of all but types B_2 and B_4 which remained about the same. The titrations after 6 passages in HeLa cells indicated higher titers in vitro than in mice for all but type B_4 virus. Furthermore, after passage in vitro there was a marked reduction in the time necessary for tissue degeneration to be manifest. Five additional passages in HeLa cells did not increase the tissue culture titers. No mouse titrations were performed with the eleventh passage materials.

Storage of the 5 virus strains in the dry ice chest for one year as undiluted tissue culture fluid (T.C.$_6$) has resulted in a decrease in the tissue culture titers of less than 1 log.

TABLE II. HYPERIMMUNE SERUM TITRATIONS IN TISSUE CULTURE NEUTRALIZATION TESTS WITH COXSACKIE B VIRUS STRAINS

SERUM DILUTION	TISSUE CULTURE DOSES OF VIRUS STRAINS NEUTRALIZED				
	TYPE B_1*	TYPE B_2*	TYPE B_3*	TYPE B_4*	TYPE B_5*
Homologous†					
1:4	> 100,000	Not done	Not done	10,000	100
1:16	10,000	10,000	1,000	100	0
1:64	4,000	1,000	100	32	0
1:128	1,000	100	32	10	0

*Virus strains employed after 6 passages in cultures of strain HeLa.

†No heterologous neutralization was noted except a 1:4 but not a 1:10 dilution of B_5 serum inhibited 1 to 10 TCD of B_4 virus.

Quantitative neutralization tests with hyperimmune sera. For determining the titers and specificity of the hyperimmune sera prepared in hamsters, as well as of the tissue culture strains, "checkerboard" titrations were performed, i.e., several dilutions of the homologous and heterologous sera were tested against several virus dilutions. Separate experiments were performed with each virus strain. Table II shows the results of homologous serum titrations. The 5 prototype sera proved to be highly specific even when heterologous serum dilutions of 1:2 or 1:4 were employed. Heterologous reactions were noted only in one instance where a 1:4, but not a 1:10, dilution of B_1 serum neutralized between 1 and 10 T.C.$_{50}$ doses of B_4 virus.

The specificity noted permitted the use of these sera for immunologic typing of isolated agents, and for routine use as standard control sera in neutralization tests. A 1:20 dilution was selected for immunologic typing except for the B_5 serum where a 1:4 dilution was employed. Immunization with this type has given consistently low serum titers in both hamsters and guinea pigs, even after a rigorous immunization schedule.

The Use of HeLa Cells in the Diagnosis of Coxsackie Infections.—The availability of strain-specific hyperimmune sera and tissue culture strains of the Coxsackie B viruses made it feasible to apply the tissue culture techniques in routine diagnosis by: (1) isolation and subsequent identification of virus from clinical specimens; and (2) identification of specific antibodies in sera of patients.

TABLE III. COXSACKIE B VIRUS ISOLATIONS FROM VARIOUS TYPES OF CLINICAL SPECIMENS IN CULTURES OF STRAIN HELA

TYPE OF SPECIMEN	NUMBER OF STRAINS ISOLATED					TOTALS FOR EACH KIND OF SPECIMEN
	B_1	B_2	B_3	B_4	B_5	
Stool	1	11	24	13	4	53
Rectal swab	0	4	3	3	0	10
Throat swab	0	6	1	10	1	18
Spinal fluid	1	0	1	0	0	2
Totals for each virus type	2	21	29	26	5	83

1. *Isolation and immunologic typing of Coxsackie B virus strains:* Tube cultures of HeLa cells employed with a variety of clinical specimens resulted in 83 isolations representing all 5 types of Coxsackie B virus (Table III). Degeneration was noted in culture tubes from 1 to 7 days following inoculation and the culture fluids were harvested and passed in tissue culture in the presence of standard hyperimmune sera for the 3 immunologic types of poliomyelitis virus and the 5 immunologic types of Coxsackie B virus, as described above under Immunologic Typing of Isolated Viruses.

Successful isolation of strains representative of 4 immunologic types of Coxsackie B virus was noted from stools, rectal or throat swabs, and from 2 spinal fluids. In one case, 2 virus types, B_2 and B_4, were isolated and in this patient neutralizing antibodies developed to B_4 virus (1:64) but were not detected to B_2 virus (< 1:16). The quantity of serum was not sufficient to employ a lower serum dilution.

Parallel attempts at virus isolation in suckling mice and HeLa cultures were made in a few instances. While the evaluation of comparative susceptibility of the two systems must await collection of more extensive data, it is apparent from the results presented in Table IV that primary isolation failed in tissue culture in some cases where the specimens yielded virus in suckling mice, and vice versa. The strains isolated in mice could be typed however, directly in tissue culture without prior passage in HeLa cells. Likewise, those strains isolated in HeLa could be passed into mice directly after isolation in tissue culture. Thus, the differential results in the two systems may represent a problem of sampling or viral concentration since the isolation in mice of Coxsackie B strains which do not readily propagate in HeLa cultures has not presented a problem to date.

TABLE IV. COMPARATIVE ISOLATIONS OF COXSACKIE B VIRUS IN SUCKLING MICE AND IN CULTURES OF STRAIN HELA

		ISOLATION RESULTS IN MICE	
		+	−
Isolation results in HeLa	+	16	5
	−	11	101

2. *Identification of specific antibodies in sera of patients.* Results of serum neutralization tests in HeLa cell cultures with paired patients' sera are presented in Table V.

Groups A1 and A2. There was a fourfold or greater increase in antibody titer to the type of virus isolated in 20 of the 25 cases studied. Five of the 20 cases showed an heterologous response as well (Group A2). Two of these 5 cases revealed heterologous titer rises to the B_1 virus strain; another 2 patients showed high heterologous titers to the B_1 virus in both acute and convalescent sera without showing a rise to this agent, and one patient demonstrated an heterologous titer to B_4 virus.

Group B. In 2 of the 25 patients a specific increase of less than fourfold was noted. From one of these patients both B_2 and B_4 viruses were isolated with an antibody response resulting to the B_4 virus only.

Group C. Two other patients had high titers against the homotypic virus in both acute and convalescent sera.

Group D. Finally, in one case, only a slight increase in titer to the homotypic virus was noted while marked increases (sixteen fold or greater) were found for two other virus types.

Although the comparative serum neutralization titrations in mice were very limited, essentially similar results were obtained with the first three pairs of sera listed in Table V. That is, significant rises in serum titer were observed only against the homotypic type B_2 agent. These titers were $< 10/40$, $20/320$, $< 20/40$, respectively. In two of these cases a slight serologic response ($< 20/20$) was evidenced against the type B_1 agent as well. Since these few neutralization tests required more than 90 litters of mice, no further comparisons were made.

DISCUSSION

The 5 immunologic types of mouse adapted Coxsackie B virus, including several strains of type B_2, were found to propagate readily in cultures of HeLa

TABLE V. COXSACKIE B SEROLOGY

GROUP	CASE NO.	TYPE VIRUS ISOLATED	DAYS AFTER ONSET SERUM COLLECTED		NEUTRALIZATION TITERS IN TISSUE CULTURE (HeLa) S_1/S_2				
			S_1*	S_2*	B_1	B_2	B_3	B_4	B_5
A1	1	B_2	3	21	< 16/ < 16	< *16/64*	< 16/ < 16	< 16/ < 16	N.D.†
	2	B_2	5	23	< 16/ < 16	< *16/64*	< 16/ < 16	< 16/ < 16	N.D.
	3	B_2	5	23	< 16/ < 16	< *16/32*	< 16/ < 16	16/16	N.D.
	4	B_2	5	23	< 16/ < 16	< *16/64*	< 16/ < 16	< 16/ < 16	N.D.
	5	B_2	5	12	< 16/ < 16	< *16/32*	< 16/16	< 16/ < 16	N.D.
	6	B_3	4	> 30	< 16/ < 16	< 16/ < 16	*16/64*	< 16/ < 16	N.D.
	7	B_3	11	25	< 16/ < 16	< 16/ < 16	*16/64*	< 16/ < 16	N.D.
	8	B_1	19	90	< *10/80-40*	< 10/ < 10	< 10/ < 10	< 10/ < 10	< 10/ < 10
	9	B_3	8	17	< 10/ < 10	< 10/ < 10	*20/80*	< 10/ < 10	< 10/ < 10
	10	B_1	1	8	30/ > 160	10/20	20/ < 10	< 10/ < 10	< 10/ < 10
	11	B_3	3	14	< 10/ < 10	< 10/ < 10	< *10/30*	10/10	< 10/ < 10
	12	B_3	6	14	< 8/ < 16	< 8/ < 16	*32/256*	< 8/ < 16	< 8/ < 16
	13	B_4	1	10	< 8/ < 16	< 8/ < 16	< 8/ < 16	*128/1024*	< 8/ < 16
	14	B_4	3	> 30	< 8/ < 16	< 8/ < 16	< 8/ < 16	< *8/512*	< 8/ < 16
	15	B_4	9	17	< 8/ < 16	< 8/ < 16	< 8/ < 16	*512/ > 1024*	< 8/ < 16
A2	16	B_2	3	12	*40/ > 80*	< *10/40*	40/60	< 10/20	< 10/ < 10
	17	B_2	< 7	15	< *10/ > 160*	< 10/ < 10	< *10/160*	< 10/ < 10	< 10/ < 10
	18	B_3	2	11	*160/160*	10/ < 10	*10/60*	< 10/ < 10	< 10/ < 10
	19	B_3	7	25	> *160/ > 160*	< 10/ < 10	< *10/40*	< 10/ < 10	< 10/ < 10
	20	B_5	7	16	< 8/ < 16	< 8/ < 16	< 8/ < 16	*128/256*	*32/256*
B	21	B_2, B_4	3	6	< 16/ < 16	< 16/ < 16	< 16/ < 16	32/64	N.D.
	22	B_4	4	20	< 8/ < 16	< 8/ < 16	< 8/ < 16	*512/1024*	< 8/ < 16
C	23	B_3	9	17	< 16/ < 16	< 16/ < 16	*64/64*	16/16	N.D.
	24	B_2	0	15	< 16/ < 16	*32/32*	< 16/16	< 16/ < 16	N.D.
D	25	B_2	6	14	*10/ > 160*	< *10/10*	*10/160*	20/20	< 10/ < 10

Positive serum titers in italics.

*S_1 = First serum sample; S_2 = second serum sample

†N.D. = not done.

cells No adaptation problems were encountered with subsequent passage in vitro Furthermore, the tissue culture system employed seemed to provide a good menstruum for primary isolation and typing of viruses of the Coxsackie B group from clinical specimens Viruses representative of all 5 immunologic types have been isolated from a variety of specimens, including 2 spinal fluids A comparison of the frequency of isolations in HeLa and in the suckling mice showed that in some instances specimens yielded virus in only one of the systems employed However, the agents so obtained could then be transmitted readily to the other host so that the failures cannot be ascribed to variant strains of virus It is obvious that for optimal results both HeLa cells and suckling mice will have to be used Virus strains, whether isolated in tissue culture or mice, could readily be identified by neutralization tests in tissue culture The tissue culture system also proved to be satisfactory for determination of neutralizing antibody responses In all of 25 cases studied there was either a rise in titer against the homotypic virus isolated (23/25) or both acute and convalescent sera showed high titers to the specific agent isolated (2/25) Where a significant heterotypic response was noted as well (6/25) cases, antibodies against the B_1 and B_4 type were involved This could possibly indicate a dual infection or an anamnestic response in the patient

SUMMARY

Cultures of HeLa cells were employed for routine isolation and immunologic typing of viruses of the Coxsackie B group in human infections Agents of all 5 types have been isolated from a variety of clinical specimens including spinal fluid Some of the agents were more readily isolated in suckling mice than in HeLa cells and vice versa The first passage strains thereafter were readily passed in either system

Serologic evidence of infection was obtained in serum neutralization tests employing tissue culture adapted viruses as antigen, and HeLa cell cultures as the host system

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STUDIES ON ERYTHROPOIESIS

VI. ERYTHROPOIETIN IN HUMAN PLASMA

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THE mechanisms which regulate erythropoiesis have been under intensive investigation in recent years. Carnot and Deflandre[1] were the first to suggest a humoral mechanism was of importance in this regulation. Investigations prior to 1952, largely confined to animals, were summarized in the *extensive review by Grant and Root,*[2] *and at that time evidence favored the* humoral control of erythropoiesis. The factor responsible for this control has been referred to as erythropoietin by Bonsdorff and Jalavisto.[3]

A significant advance was made by Borsook, Graybiel, Keighley, and Windsor[4] when they demonstrated an erythropoietic stimulating factor in the filtrate obtained from heat denatured anemic rabbit plasma. Although obtained from anemic rabbits, this filtrate could be assayed in the normal rat. The appearance of an erythropoietic factor in the plasma of rats or rabbits made anemic by phlebotomy or injection of phenylhydrazine has been demonstrated by numerous investigators.[5-9]

Recent investigators have turned their attention to the significance of erythropoietin in the human being. Contopoulos and his co-workers[10] have demonstrated a plasma factor from blood of patients with polycythemia vera which is capable of stimulating erythropoiesis in the rat. Linman and Bethell[11] have demonstrated stimulation of erythropoiesis in the normal rat by injections of extracts of plasma from patients with polycythemia vera and secondary polycythemia. Pilero and co-workers[12] have found an erythrocyte stimulating factor in the plasma of patients with Cooley's anemia and sickle-cell anemia. Their report also summarizes previous work with human plasma by other investigators.

The purpose of this report is to record the demonstration of erythropoietin in the extract of heat denatured plasma obtained from anemic patients. This demonstration has been facilitated by the development of an assay procedure by Plzak, Fried, Jacobson, and Bethard[13] dependent on the increased incorporation of radioiron into erythrocytes of rats stimulated by the intravenous injection of erythropoietin. Although Gordon, Piliero, Tannenbaum, and Siegel[14] had previously demonstrated that the hypophysectomized rat was capable of responding to anemic rabbit plasma extract with increases of peripheral red cell, hemoglobin, hematocrit, and reticulocyte values, Jacobson and his co-workers[15, 16] first clearly demonstrated that the hypophysectomized

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rat was an extremely sensitive animal for assay of erythropoietin activity and that a dramatic increase of Fe^{59} incorporation into the animal's erythrocytes followed only 2 or 3 injections of anemic rat plasma. The significance of this assay method rests in the great increase of red cell Fe^{59} incorporation, often several hundred per cent greater than that produced by normal plasma, requiring only small amounts of anemic plasma. It has therefore become possible to avoid lengthy assays in which small rises in peripheral hemoglobin, red count, or hematocrit following many injections of a plasma extract represent activity of the extract being tested. In the present study this assay procedure was used in an attempt to determine the presence of erythropoietin in normal and anemic human plasma.

METHODS

Fifty to 150 ml. of blood from anemic patients was collected in 250 ml. bottles each containing 1 ml. (1,000 units) of heparin. Normal control blood was collected in 500 ml. bottles each containing 2 ml. of heparin. Immediately after collection the plasma was separated by centrifugation in the cold, removed, and stored in the Deepfreeze until extraction.

Plasma was extracted by the boiling method as described by Borsook and co-workers.[4] The plasma was brought to a pH of 5.5 by addition of 0.1 normal hydrochloric acid, put into a boiling water bath for 10 minutes, and filtered. The precipitate was washed twice with an equal volume of distilled water, all filtrates being pooled. Employing a rotary evaporator, the filtrate was then concentrated to the volume of the original plasma specimen. Since normal plasma failed to demonstrate erythropoietic stimulating activity, we considered the possibility that erythropoietin might be present in titers insufficient to demonstrate in normal plasma by the assay system employed and some specimens of normal plasma were, therefore, concentrated to one-tenth the original volume. Such specimens were then dialyzed for 48 hours in order to remove the excessive amounts of electrolytes present as a result of concentration. After concentration to the desired volume, the pH was adjusted to 7.5 by the addition of 0.1 normal sodium hydroxide.

An extract of normal plasma was also prepared using perchloric acid. A volume of 10 per cent perchloric acid equal to the plasma specimen was added to the plasma slowly with continuous stirring. The precipitate was washed two times with 5 per cent perchloric acid, *each washing was with a volume equal to one-half the original plasma volume*. The supernatant fluid and washings were placed in a Visking Casing bag, dialyzed against distilled water for 72 hours, concentrated to the desired volume, and the pH was adjusted to 7.5 by the addition of 0.1 N sodium hydroxide.

Hypophysectomized male Sprague-Dawley rats, 130 to 180 grams in weight, were used. The animals in each assay were 8 to 15 days posthypophysectomy when the assay was begun. On 2 successive days 2 ml. of anemic plasma extract were injected in the tail vein while the animals were under light ether anesthesia. On the third day a tracer dose of 1 to 2 μc of Fe^{59} (activity approximately 2,000 mc per gram of iron) in saline, buffered to pH 7.5 with sodium citrate was injected. Sixteen hours after iron injection, 1 ml. of blood was obtained by cardiac puncture for assay. Between successive cardiac punctures, the syringe used was rinsed in normal saline and finally in heparin (1,000 units per milliliter). The thin film of heparin left in the syringe was sufficient to prevent clotting.

Blood samples and two aliquots of the standard prepared at the time of radioiron injection were counted in a Nancy Wood scintillation counter. Assuming a blood volume of 5 per cent of body weight[13] and correcting counts for background, the per cent Fe^{59} incorporation in red cells at 16 hours was calculated. Per cent iron incorporation =

$$\frac{\text{net counts of blood per minute/ml.} \times \text{body weight in grams} \times 0.05}{\text{net counts of standard/ml.}}$$

Results are expressed as average per cent uptake ± one standard deviation. Starved, normal, male Sprague-Dawley rats of the same age and weight as the hypophysectomized animals were also occasionally used for assay.[17] These animals had no food for 24 hours prior to the beginning of the assay, and were maintained in the starved condition throughout the assay, water being given ad libitum.

At least one normal plasma control and a saline control assay was run every 2 weeks over the duration of the entire study. A normal plasma control was not, however, run with every assay.

RESULTS

Studies on Normal Plasma.—The average uptake obtained when the extracts of individual heat denatured plasma specimens from 10 normal donors with hemoglobin above 14 Gm. per cent was used in hypophysectomized rats was 3.1 per cent; this value does not differ appreciably from an uptake of 3.4 per cent when normal saline was used. In starved rats, 3.5 per cent uptake was obtained with extract of normal plasma from 6 donors, and an uptake of 3.2 per cent followed the injection of normal saline. These results are summarized in Table I. It seemed likely to us that normal plasma was not devoid of erythropoietin, but that the concentration was so small as to escape detection by this assay. To test this possibility, after precipitation of proteins by heat or perchloric acid, the supernatant fluid was concentrated 10 times. The incorporation of Fe^{59} into red cells was sufficiently increased, when compared to incorporation following injection of saline or unconcentrated plasma extracts to indicate that erythropoietin is present in normal plasma. These results are summarized in Table II.

TABLE I. INFLUENCE OF EXTRACT OF NORMAL HUMAN PLASMA ON PER CENT OF Fe^{59} INCORPORATION IN RAT ERYTHROCYTES*

Assay of heat denatured plasma from 10 normal human donors assayed in 49 hypophysectomized rats	3.1 ± 1.1%
Saline control in 40 hypophysectomized rats	3.4 ± 1.4%
Assay of heat denatured plasma from 5 normal human donors assayed in 22 hypophysectomized rats	3.5 ± 1.7%
Saline control in 25 starved rats	3.2 ± 1.1%

*Average values ± one standard deviation.

TABLE II. ERYTHROPOIETIC ACTIVITY OF FRACTIONATED CONCENTRATED NORMAL PLASMA (5 Hypophysectomized Rats in Each Group)

Heat denatured	10 × normal concentration, dialyzed	12.6 ± 2.1%
	Normal concentration, dialyzed	2.6 ± 1.2%
Perchloric acid	10 × normal concentration, dialyzed	7.6 ± 2.5%
	Normal concentration, dialyzed	2.8 ± 1.3%

Assay of Plasma From Anemic Patients.—Twenty-one plasma specimens were obtained from 18 patients with anemia. Twelve were positive, and 9 were considered equivocal or negative. At this time, we prefer to consider only those assays averaging 7 per cent or more in hypophysectomized rats as positive (control = 3.1 per cent), although undoubtedly lower values could be considered positive if larger numbers of animals were used in each assay. Results of these assays are summarized in Table III.

In the first patient, hypoplastic anemia was secondary to Atabrine which had been administered on 3 occasions for treatment of chronic discoid lupus

and 9 were positive In keeping with our arbitrary assumption that incorporation of 7 per cent or more of the administered dose is required before an assay be considered positive, plasma from Patient 10 is classified as negative, although 6 3 per cent incorporation clearly exceeds any of the control values Patient 11 had chronic granulocytic leukemia accompanied by mild anemia Assays of extracts of his plasma were negative in both hypophysectomized and starved rats

Positive results were obtained when extracts of plasma from patients with pernicious anemia, acute gastrointestinal hemorrhage, and Hodgkin's disease with anemia were assayed

Diagnoses of patients whose plasma extract was negative or equivocal are enumerated in Table III Only Patients 11 and 16 were in a good state of nutrition Patient 17 was in a poor state of nutrition and has persistently in the last year had a hemoglobin under 9 Gm The remaining patients were cachectic and considered to be approaching terminal status at the time blood was obtained for assay

DISCUSSION

The rats used for assay in this study have plasma volumes of approximately 3 5 ml Therefore, it is logical to inquire as to why the erythropoietin in 4 ml of normal plasma is not sufficient to stimulate erythropoiesis Although we cannot answer this question at the present time, we believe there is loss of activity of plasma after removal from the body[19] We are not now able to ascertain exactly how much erythropoietin is lost in the extraction employed here, but preliminary studies in our laboratories suggest about 50 per cent loss[19] If the rate of in vivo turnover is great, it may be that several times that amount of erythropoietin present in the normal rat at any one time would have to be given to the hypophysectomized or starved rat over a 2 day period in order to increase erythropoiesis And finally, if under physiologic circumstances the rat should have a higher concentration of erythropoietin than the human, concentrations which would be normal for the latter would not be sufficient to produce an optimal response in the rat

It is apparent that without concentrating the plasma extract, an active erythropoietic stimulating principle can be demonstrated in the heat denatured extract of plasma from human beings with a variety of anemias It is not entirely clear at this time why a high titer of erythropoietin cannot be demonstrated in the plasma of all anemic subjects A recent abstract by Prentiss and Mirand[20] reports demonstration of erythropoietic activity in plasma of 10 to 15 patients with anemia, using the assay procedure first employed by Fried, Plzak, Jacobson, and Goldwasser[16] The diagnoses associated with the anemias were not stated In their study the extract was concentrated 3 times It is probable that some of our extracts which were negative would have been positive had the plasma been concentrated We chose not to concentrate plasma extracts in this initial clinical study for two reasons First, we have been reluctant to remove routinely more than 50 ml of blood from severely anemic patients until we had thoroughly evaluated the results we could obtain by assaying a series of anemic plasmas without concentrating them Second, if it is to give the most meaningful results, concentration of anemic plasma should be as many times as is required to demonstrate activity in normal, nonanemic plasma. Although we have demonstrated activity in normal plasma extracted by perchloric acid precipitation or heating and concentrated 10 times, we have not yet determined in a large series of normal plasma specimens the minimum number of times the filtrate from heat denatured normal plasma must be concentrated in order to demonstrate activity. Concentration of extracts of anemic plasma an arbitrary number of times may yield a few more positive assays. It will not, however, make negative results any more meaningful. Negative assays will be significant only when they indicate decreased concentrations of erythropoietin in plasma of anemic subjects. We have chosen not to concentrate extracts of anemic plasma until we have determined the number of times we must concentrate them in order to make a negative result indicative of decreased erythropoietin concentrations.

It may be, however, that failure to demonstrate erythropoietin in the plasma of all anemic patients cannot in every instance be attributable to insensitivity of the assay method. It appears possible to us that some anemic states may be accompanied by and to some degree attributable to a decreased concentration of erythropoietin, with a new steady state being established at hemoglobin levels less than normal.

Bethard and Wissler[21] have demonstrated reduction of the rate of erythropoiesis in protein depleted rats as measured by Fe^{59} uptake, and Fried, Plzak, Jacobson, and Goldwasser[17] have reported a decreased rate of erythropoiesis in acutely starved animals. It may be that in some patients, as in Patients 14 and 15 with carcinoma, starvation contributes to decreased erythropoiesis by decreasing erythropoietin production. Finally, in light of recent evidence reported by Jacobson and associates[22] suggesting that erythropoietin is produced in the kidney, it is interesting to speculate that decreased production of erythropoietin may have contributed to failure to demonstrate activity in the extract of anemic plasma of a patient with chronic renal disease. Excessive excretion of erythropoietin in chronic renal disease is still another possibility which warrants investigation, particularly since an erythropoietic stimulating factor has been demonstrated in the urine of an anemic patient by Piliero and co-workers.[12]

The appearance of increased concentration of erythropoietin in animals following bleeding or hemolysis,[1-9, 13-16] the high concentrations of erythropoietin in several of our patients, and the prompt disappearance of the high concentration in one patient following blood transfusion suggests to us the possibility that the homeostatic mechanism by which a patient responds to anemia by increased erythropoiesis is mediated through production of increased amounts of erythropoietin. If future investigation substantiates this hypothesis, it is apparent the assay method as now employed is not very sensitive, since reticulocyte counts as high as 8.9 per cent were found in patients whose plasma failed to demonstrate activity on bio-assay.

It is clear that a relationship does not of necessity exist between severity of anemia and erythropoietin concentration or degree of reticulocytosis and erythropoietin concentration. A hemoglobin of 5.2 is associated with a plasma erythropoietin concentration sufficient to increase Fe^{59} incorporation sixfold in the case of hypoplastic anemia, while the plasma of a nephritic patient with a comparable anemia failed to demonstrate activity by the method employed. The bone marrow may be refractory to the stimulus produced by an increased erythropoietin content, as in Patient 5 with leukemia where no reticulocytes were present, while levels of erythropoietin sufficiently below the threshold of detection by this method may still be associated with a reticulocytosis, as in Patients 1, 11, and 18.

We are led to postulate that patients with severe anemia have a high plasma titer of erythropoietin unless its production is decreased or its destruction or excretion is increased. In those instances where the patient will spontaneously correct the low hemoglobin levels, as following acute hemorrhage and drug induced hemolytic anemia, we believe the elevated erythropoietin concentration will be found to decrease as the hemoglobin rises. The erythropoietin content may be expected to remain high in anemias which are not correctable by the unaided homeostatic body mechanisms, but will probably be found to decrease following blood transfusion to a normal level or following rise of hemoglobin to a normal level, as in pernicious anemia adequately treated with B_{12}. Finally, we believe future work may indicate some anemias will be attributable to decreased or absent erythropoietin production. The recent investigation of Pilero and co-workers[12] suggests primary red cell anemia of children may be such a disease. Since cobalt appears to act by increasing the production of erythropoietin,[13] it is reasonable to anticipate anemias associated with renal disease, chronic infection, neoplasm, and rheumatoid arthritis, all responsive to cobalt administration[14-18] may be attributed to low plasma erythropoietin concentrations and may, therefore, respond to erythropoietin when a preparation sufficiently potent and pure enough to permit clinical trials becomes available. The decreased rate of iron incorporated in the red cells of starved rats and the response in these animals to anemic rat plasma[17] suggest anemias accompanying starvation and cachectic states may be at least partially the result of low plasma erythropoietic concentrations.

SUMMARY

1. Using the incorporation of Fe^{59} into erythrocytes of hypophysectomized or starved male Sprague-Dawley rats, erythropoietin has been demonstrated in normal plasma. This required tenfold concentration when plasma extracts are prepared by heat denaturization or perchloric acid precipitation.

2. Without concentrating the plasma extracts, erythropoietin has been demonstrated in heat denatured plasma extracts of 10 anemic subjects.

3. Hypoplastic anemia, in 3 patients, cannot be attributed to decreased erythropoietin concentration.

4. High erythropoietin concentrations in the blood of one patient with hypoplastic anemia fell promptly following blood transfusion.

5. Failure to demonstrate activity by this method is not necessarily indicative of decreased erythropoietin concentrations.

6. The results of this study are consistent with the hypothesis, first formulated by Carnot and Deflandre in 1906, that the rate of erythropoiesis is mediated by a humoral mechanism.

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OBSERVATIONS ON THE NATURE OF THE ERYTHROPOIETIC SERUM FACTOR

II. Erythropoietic Activity of Serum and Bone Marrow After Time Limited Exposure to Anemic and Hypoxic Anoxia

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THE rate of red cell production, although conditioned by many known minerals, vitamins, and hormones, appears to be controlled by the tissue tension of oxygen. The physiologic mechanism responsible for this control is unknown. However, it has been suggested that a humoral erythropoietic factor might serve as a mediator between a specific oxygen sensitive tissue and the bone marrow.[1]

Recently, this concept has received experimental support by the demonstration of an erythropoietic factor in plasma and serum from animals exposed to anemic and hypoxic anoxia (see review by Erslev[2]) The erythropoietic factor has not been isolated or purified as yet, but it has been found to be present in a nondialyzable fraction of the protein-moiety of serum.[3]

In order to define further the physiologic mechanism of the control of erythropoiesis, the time relationship between the induction of tissue anoxia and the appearance and persistence of erythropoietic factor in serum was studied.

METHODS

Adult, white male New Zealand rabbits weighing 2.2 to 3.2 kilograms were used throughout Anemic anoxia was induced by a single bleeding of 20 ml. per kilogram body weight. In order to minimize circulatory adjustment, the bleeding was promptly followed by an intravenous infusion of an equal volume of 6 per cent dextran in saline. The anemia was terminated by reinfusion of the blood removed for which heparin was used as anticoagulant.

Hypoxic anoxia was induced by exposing the animals to 10 or 11 per cent oxygen at normal barometric pressure in an airtight chamber.[4] The oxygen level was kept constant by admitting compressed room air and pure nitrogen at flow rates sufficient to provide a total air flow per minute of 4 liters per animal. An electric fan maintained even oxygen concentration in the chamber, while calcium chloride and calcium hydroxide removed excessive moisture and carbon dioxide.

The serum obtained within a few minutes after the termination of the anoxic periods was bio assayed for erythropoietic factor. For each bio assay 3 to 4 animals were exsanguinated and their serum pooled. The technique used in the bio assay has been described in detail in a previous publication [3] In short, 50 ml of the serum to be tested was

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infused intravenously into normal rabbits once a day for 4 days. Reticulocyte counts and hemoglobin concentrations were followed for 7 days, and the magnitude of the reticulocyte response was taken as an index of the erythropoietic activity of the infused serum.

Reticulocyte counts were made from blood smears on cover glasses, prepared with brilliant cresyl blue. The smears were counterstained with Wright's Stain. One thousand red cells were counted. During anemic periods the reticulocyte percentages were corrected for the fall in the total number of red cells, and expressed in per cent of the initial red cell count.

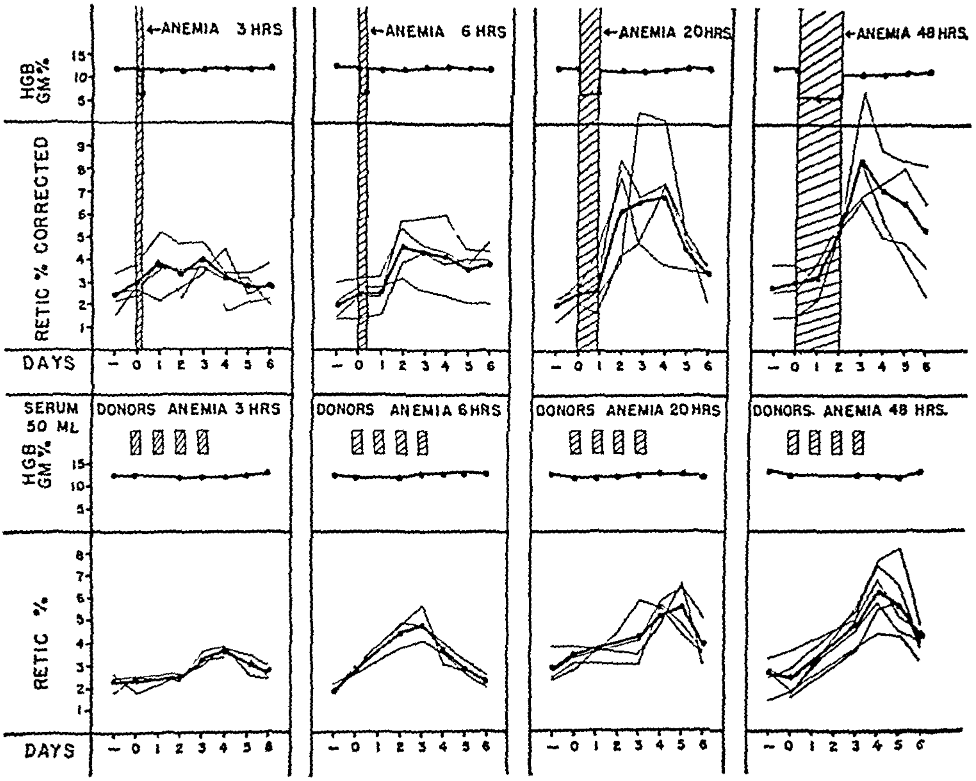

Fig 1—Charts in upper row Mean hemoglobin concentrations and mean and individual reticulocyte percentages of normal rabbits bled 20 ml per kilogram with immediate restoration of blood volume by 6 per cent dextran in saline In different groups of rabbits the anemia was terminated 3, 6, 20, or 48 hours later by reinfusion of the previously removed blood During the anemic periods reticulocyte percentages were corrected for the decrease in the number of red blood cells Charts in lower row Mean hemoglobin concentrations and means and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml of serum Serum was obtained from groups of rabbits after 3, 6, 20, or 48 hours of anemic anoxia

RESULTS

1. *Reticulocyte Response to Anemic Anoxia.*—Groups of normal rabbits were rendered anemic by blood loss (Hgb $<$ 7.0 Gm. per cent) for 3, 6, 20, or 48 hours After such intervals the anemia was terminated by reinfusion of the blood drawn The individual and mean reticulocyte responses to this acute, time-limited anemia are charted in the upper portion of Fig. 1, along with the mean hemoglobin concentrations A moderate increase in the number of reticulocytes was observed in most animals kept anemic for 6 hours. Anemia maintained for 20 hours induced a significant reticulocytosis, although, less pronounced than the response observed after 48 hours of anemia.

2. *Erythropoietic Activity of "Anemic" Serum.*—Groups of rabbits rendered anemic by blood loss (Hgb. < 7.0 Gm. per cent) for 3, 6, 20, or 48 hours were bled to death and the serum from each group bio assayed for erythropoietic activity. The results are shown in the lower portion of Fig. 1. Serum from animals kept anemic for 3 hours was only questionably active, while serum from animals kept anemic for 6 hours showed a definite erythropoietic activity. Even greater activity was present in serum from animals kept anemic for 20 hours and 48 hours. Serum from animals with a hemoglobin concentration maintained at less than 7 Gm. per cent for 5 days, revealed erythropoietic activity similar to the activity found after 20 and 48 hours of anemia (Fig. 2, *A*).

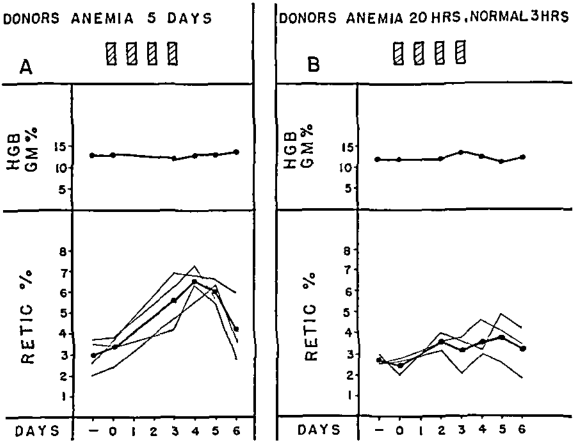

Fig. 2.—Mean hemoglobin concentrations and mean and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml. of serum. *A*, Serum obtained from rabbits after five days of sustained anemia (Hemoglobin < 7.0 Gm. per cent). *B*, Serum obtained from rabbits 3 hours after the termination of a 20 hour period of anemic anoxia.

3. *Erythropoietic Activity of Serum After Termination of Anemic Anoxia.*—Rabbits rendered anemic by blood loss (Hgb. < 7.0 Gm. per cent) for 20 hours, were bled to death 3 hours after the anemic anoxia had been terminated by means of reinfusion of the previously removed blood. Bio assay of this serum revealed an almost complete disappearance of erythropoietic activity (Fig. 2, *B*).

4. *Reticulocyte Response to Hypoxic Anoxia.*—Groups of normal rabbits were exposed to 11 per cent oxygen for 3, 6, or 20 hours and their erythropoietic response was compared with results obtained in a previously reported study, with rabbits exposed to 10 per cent oxygen for 48 hours.[4] As shown in the upper portion of Fig. 3 no reticulocyte response followed 3 and 6 hours of hypoxia. Hypoxia lasting 20 hours and 48 hours were followed by a definite

increase in the number of reticulocytes. However, comparison with Fig. 1 shows that the response to hypoxic anemia in each instance was less than the response to anemic anoxia of corresponding duration.

5. *Erythropoietic Activity of "Hypoxic" Serum.*—Groups of rabbits exposed to 11 per cent oxygen for 3, 6, or 20 hours and another group of rabbits exposed to 10 per cent oxygen for 48 hours[4] were bled to death and the sera from each group of animals were bio-assayed for erythropoietic activity. As shown in the lower portion of Fig. 3 the activity of these sera corresponded closely to that of sera from rabbits kept anemic for corresponding periods of time.

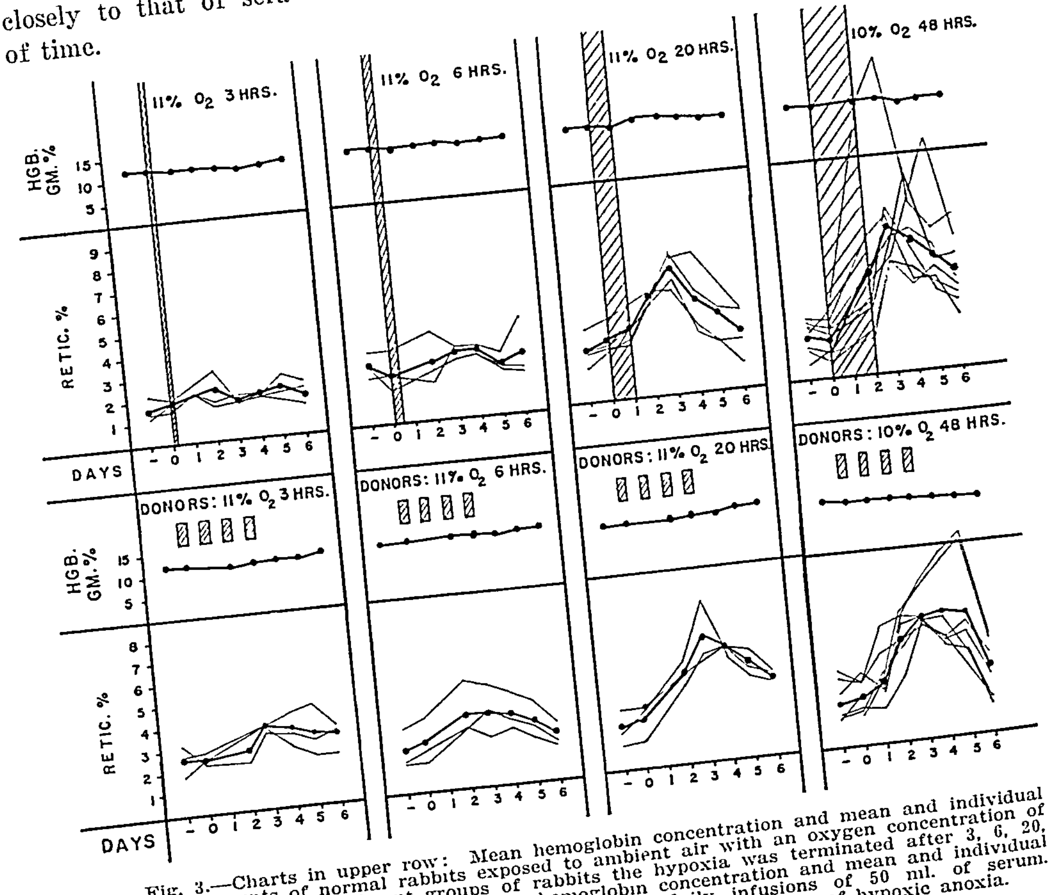

Fig. 3.—Charts in upper row: Mean hemoglobin concentration and mean and individual reticulocyte counts of normal rabbits exposed to ambient air with an oxygen concentration of 10 or 11 per cent. In different groups of rabbits the hypoxia was terminated after 3, 6, 20, or 48 hours. Charts in lower row: Mean hemoglobin concentration and mean and individual reticulocyte counts of normal rabbits receiving four daily infusions of 50 ml. of serum. Serum was obtained from groups of rabbits after 3, 6, 20, or 48 hours of hypoxic anoxia.

DISCUSSION

A reduction in the oxygen carrying capacity of blood, or a reduction in the oxygen saturation of its hemoglobin will result in a decrease in the tissue tension of oxygen.[5] Under physiologic conditions, this decrease is associated with an increased rate of red cell production and with the appearance of erythropoietic factor in the serum.

In the study reported here, an acute anemic anoxia with a reduction in hemoglobin concentration to approximately 50 per cent of normal was induced by means of a single bleeding and maintained for 3, 6, 20, or 48 hours. Changes in the number of reticulocytes during the next 6 days were used to evaluate the effect of these periods of anoxia on the bone marrow. A slight increase in erythropoietic activity was noticeable after 3 hours of anemic anoxia, with a further progressive increase as the periods of anoxia were prolonged. The reticulocyte responses occurred after a delay of 48 hours without evidence of a significant immediate release of reticulocytes.

Erythropoietic activity of serum obtained at the end of the anoxic periods was found to be slight, but measurable after 3 hours, was definite after 6 hours and very significant after 20 hours of anemic anoxia. Anemic anoxia maintained for 48 hours or for 5 days, was associated with the same degree of erythropoietic activity of serum, as after 20 hours. After termination of the anemic periods, the erythropoietic activity of serum was found to have almost completely disappeared in three hours.

From these data, it is impossible to state definitely whether the appearance of erythropoietic activity in serum precedes increased bone marrow activity, or whether the erythropoietic factor merely reflects increased mitotic activity of the normoblastic tissue in the bone marrow. However, it has been shown that anemic anoxia in animals that have received nitrogen mustard is associated with erythropoietic activity of the serum, despite complete absence of erythropoietic activity of the bone marrow.[3] Consequently, it seems likely that erythropoietic activity of serum precedes erythropoietic activity of the bone marrow. This would support the hypothesis that tissue anoxia controls the production or release of an erythropoietic factor, which in turn controls the rate of red cell production. Further support for this hypothesis comes from the observation that erythropoietic activity of serum, induced by anemic anoxia, disappears rapidly after correction of the anemia.

Acute hypoxic anoxia was induced by exposing animals to air mixtures containing 10 or 11 per cent of oxygen. Hypoxic anoxia lasting 3 and 6 hours was not followed by any significant increase in the number of reticulocytes. Hypoxic anoxia of 20 hours' duration was followed by a definite reticulocyte response, although less than the response observed[4] after 48 hours of hypoxic anoxia. The increase in erythropoietic activity of the bone marrow was less in all groups than the increase observed after anemic anoxia of a corresponding duration. This difference in response might be due to a difference in the degree of tissue anoxia. It is very possible that a 50 per cent reduction in hemoglobin concentration will result in a more severe tissue anoxia than a 50 per cent reduction in the oxygen concentration of ambient air. However, the anoxic insult in both the anemic animals and in the hypoxic animals was close to the limit of tolerance, with a 10 to 15 per cent mortality. Furthermore, the erythropoietic activity of sera obtained immediately after corresponding periods of hypoxic anoxia and anemic anoxia were strikingly similar. These considerations suggest that the degree of anoxia in the anemic animals and in the hypoxic animals were approximately the same. The difference in bone marrow response could conceivably be related to the fact that compensatory hyperventilation and alkalosis occur in animals with hypoxic anoxia, and not in animals with anemic anoxia.

The tissue or organ in which a low tissue tension of oxygen causes an elaboration of the erythropoietic factor is still unknown. Saline extracts from various tissues including bone marrow, spleen, liver, thymus, lung, and kidneys obtained from anemic animals have not been shown to be erythropoietically active.[6]

Pituitary extracts have been shown to possess some erythropoietic activity.[7, 8] However, it appears unlikely that the erythropoietic factor is produced in the pituitary gland, since this factor has been found in the serum of hypophysectomized animals exposed to anemic anoxia.[9, 10] Recently, Jacobson and co-workers[11] have suggested that the erythropoietic factor is produced in the kidneys. Unfortunately, observations on uremic rabbits do not support this attractive idea.[12] First, serum from rabbits rendered both anemic and uremic showed a low level of erythropoietic factor whether the uremia was induced by bilateral nephrectomy or by bilateral ureter ligation. Furthermore, the bone marrow of nephrectomized rabbits continued to be active despite the complete absence of kidney tissue. That the factor is not elaborated in the blood stream itself is suggested by the fact that plasma from normal whole blood, equilibrated in vitro against pure nitrogen for 24 hours, failed to show erythropoietic activity. Likewise, plasma from whole blood of anemic animals remained erythropoietically active despite equilibration against air mixtures with 21 per cent oxygen and 100 per cent oxygen for 24 hours.[6]

That some specific tissue system must be responsible for the elaboration of the erythropoietic factor seems obvious, but much more work and probably a less laborious bio-assay technique will be necessary before this system and the physiologic mechanism responsible for the production of erythropoietic factor can be identified.

SUMMARY

Anemic anoxia with a 50 per cent reduction in hemoglobin concentration or hypoxic anoxia with a 50 per cent reduction in the oxygen tension of ambient air were induced and maintained in groups of normal rabbits for 3, 6, 20, or 48 hours, respectively. The reticulocyte response to the anoxia was determined in each animal, as well as the erythropoietic activity of serum obtained at the end of the anoxic periods.

In animals with anemic anoxia a moderate increase in the number of reticulocytes was found after 6 hours of anemia, with progressively greater increases in the reticulocyte response after 20 and 48 hours of anemia. In animals with hypoxic anoxia there was no response after 3 and 6 hours of hypoxia, while 20 and 48 hours induced significant reticulocyte responses. However, these were lower than in the animals kept anemic for corresponding durations of time.

The erythropoietic activity of the pooled serum obtained at the end of the anemic periods was found to be questionable after 3 hours, definite after 6 hours, and maximal after 20 hours Maintenance of the anemic state for 48 hours or for 5 days did not change the activity of the serum The erythropoietic activity of serum from animals with hypoxic anoxia corresponded closely to the activity of serum from rabbits kept anemic for the same length of time

Serum obtained 3 hours after the termination of a 20 hour period of anemic anoxia was found to have only minimal erythropoietic activity

I wish to thank Miss Elva Ruiz for valuable assistance in this study

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NEONATAL SEROLOGIC DIAGNOSIS OF HEMOLYTIC DISEASE OF THE NEWBORN CAUSED BY ABO INCOMPATIBILITY

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THE diagnosis of hemolytic disease of the newborn (HDN) is considerably more difficult if the disease is caused presumably by maternal isosensitization to the A or B antigen than when Rh incompatibility is responsible. In addition to the fact that most cases of AB-hemolytic disease are clinically mild, we are faced with a more complex serologic situation than is the case in HDN caused by sensitization to the Rh factor or even to other, more rarely involved, blood factors. In all other cases of HDN, detection in maternal serum of the antibody for a blood factor present in the infant's red cells represents prima facie evidence, whereas in HDN caused by AB incompatibility investigation of the maternal serum is hardly ever conclusive: physiologic or so-called "natural" isoagglutinins for the red cells of the infant are always present. Consequently, immunologic studies must be used to demonstrate quantitative and, if possible, qualitative changes in the antibody, especially the presence of "immune" or incomplete anti-A or anti-B. For the purpose of this presentation, complete isoagglutinins are defined as antibodies capable of agglutinating saline-suspended red cells, whereas incomplete isoagglutinins are antibodies not capable of doing so, but require the use of the indirect antiglobulin technique and/or papain-treated red cells for their detection.

The interpretation of results of prenatal and neonatal studies of maternal serum is further complicated by the fact that relatively high titers of incomplete ("immune") isoagglutinins may be found in ABO-heterospecific pregnancies that are not associated with HDN. Finally, neonatal diagnosis on the infant's blood present a further difficulty: in contrast to HDN caused by Rh and other isoantibodies, the direct antiglobulin (Coombs') test on the red cells of the infant is only rarely positive. In those rare cases the reaction is feeble, and requires special techniques for demonstration.

These circumstances have prompted many authors to investigate the possibility of finding some other method of diagnosis of the disease. Pertinent literature will be reviewed later.

The primary purpose of the present study was to test for the presence of the incompatible agglutinin in cord bloods of normal infants, as well as of infants suspected of or diagnosed as having HDN attributable to ABO incompatibility. We assumed that the situation in ABO incompatibility would be

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similar to that in Rh incompatibility, namely, that the offending antibody must traverse the placental barrier and hence appear in the infant's blood. In analogy to Rh sensitization, we assumed that incomplete antibodies would be probably associated with presence of disease A preliminary report of this work was presented in 1956.[1]

MATERIAL AND METHODS

Most specimens tested were cord blood obtained at the time of delivery from presumably normal infants The specimens were collected as clotted blood Although heterospecific pregnancies were of primary interest, cord bloods from homospecific pregnancies were also tested. Some of the specimens, especially those of infants suspected of or diagnosed as having HDN, were venous blood.

ABO and Rh types were determined within 24 to 48 hours Serum was separated from the red cells as soon as possible, and, if not tested immediately, was stored frozen at –20° C Most tests were carried out with specimens not more than 1 to 2 days old Blood groups and Rh types of the mothers were available from prenatal tests, and rechecked whenever indicated

Isoagglutinins were tested for by volumetric titrations in which 0 1 ml. of the red cell suspension was added to 0.1 ml of serum and of twofold serial dilutions of the serum The following techniques were employed:

1. Mixtures of 2 per cent saline suspended red cells and serum dilutions were centrifuged immediately (30 seconds in a small table centrifuge, commercially available as "Serofuge"[2]), and the readings recorded.

2 The tubes used in Technique 1 were shaken gently, incubated for 2 hours in a water bath at 37° C, read, and the results recorded

3 Tubes not showing agglutination were converted into indirect antiglobulin tests

4. In another set of titrations, papain treated red cells[3] were added to the serum and serum dilutions and incubated at 37° C. for ½ hour

5. Some specimens were also tested in a macromolecular medium, by using 2 per cent suspensions of red cells in 30 per cent bovine albumin and AB serum for dilution of the serum. Since this procedure did not produce agglutination as frequently as the other methods, it was omitted from the latter part of the study.

All tests were carried out with pooled fresh cells obtained from at least two individuals of groups A_1, B, and O The latter cells were included as control for possible detection of nonspecific agglutinins

RESULTS

Incompatible agglutinins were found in 6 of the 48 combinations of mother O-baby A, in 3 of the 10 combinations of mother B-baby AB, in 4 of the 27 combinations of mother O-baby B, and in one of the 11 combinations of mother A-baby AB No instance of incompatible agglutinins was found among the other combinations In 4 of the 14 cord bloods with incompatible agglutinins the antibody was complete. Clinical and hematologic examination of the 14 infants with incompatible agglutinins failed to disclose evidence of hemolytic disease

In all 34 cases of hemolytic disease of the newborn, in which the presumptive diagnosis was first made on the basis of clinical and hematologic findings, incompatible incomplete agglutinins were present (Table I). In most instances both the antiglobulin technique and the test with enzyme-treated red cells gave positive results, but in 5 instances only the test with papain-treated red cells, and in 4 only the antiglobulin test were positive. With either method the titers were low, rarely showing agglutination in serum dilutions higher than 1:2.

TABLE I. INCOMPATIBLE AGGLUTININS IN INFANTS WITH HDN CAUSED BY ABO INCOMPATIBILITY

BLOOD GROUP		NO. OF CASES	METHOD CAPABLE OF DETECTING					
			ANTI-A			ANTI-B		
MOTHER	INFANT		AGT	PAP.	AGT + PAP.	AGT	PAP.	AGT + PAP.
O	A	26	4	3	19			
B	AB	1		1				
O	B	5					1	4
A	AB	2						2
	Total	34	4	4	19		1	6

AGT = Antiglobulin technique.
PAP. = Papain-treated red cells.

In order to compare and evaluate the transplacental transfer of complete and incomplete anti-A and anti-B isoagglutinins, cord blood of homospecific pregnancies was also tested. Homospecific pregnancies are defined as those in which the mother does not possess isoagglutinins capable of agglutinating the red cells of her infant, whereas in heterospecific pregnancies mothers have such isoagglutinins. In Table II the findings in 106 homospecific and in 123 heterospecific pregnancies are summarized. In accord with previous investigations,[4-6] the highest percentage of infants with isoagglutinins demonstrable in cord blood was in those born to mothers of group O.

TABLE II. COMPLETE AND INCOMPLETE ISOAGGLUTININS IN CORD BLOOD OF NORMAL INFANTS

	BLOOD GROUP		NO. OF CASES	PRESENCE OF			
				ANTI-A		ANTI-B	
	MOTHER	INFANT		COMPLETE	INCOMPLETE	COMPLETE	INCOMPLETE
Homospecific pregnancies	O	O	29	26	3	26	2
	A	A	29			9	11
	A	O	18			8	0
	B	B	18	9	6		
	B	O	7	4	2		
	AB	A, B, AB	5				
		Total	106	39	11	43	13
Heterospecific pregnancies	O	A	48	1*	5*	34	8
	O	B	27	24	3	1*	3*
	A	B, AB	24				1*
	B	A, AB	24	2*	1*		
		Total	123	27	9	35	12

*Incompatible agglutinin.

The data shown in Table II lend themselves to an analysis of the relationship between the presence of isoagglutinins in mothers and their normal infants, exclusive of incompatible agglutinins. The results of this analysis are summarized in Table III. When anti-A was present in the mother, it was absent in less than 5 per cent of the compatible infants, whereas maternal anti-B was absent in 21 per cent of infants not possessing the B antigen. This difference in behavior of anti-A and anti-B antibodies appears to be primarily the result of the less frequent passage of complete anti-B agglutinins as compared with anti-A, while transplacental transfer of incomplete antibodies is approximately the same for both antibodies.

TABLE III. RELATIONSHIP BETWEEN ISOAGGLUTININS IN MATERNAL AND CORD BLOODS IN NORMAL INFANTS*

ISOAGGLUTININ PRESENT IN MATERNAL SERUM	TYPE OF PREGNANCY	NO. OF CASES	ISOAGGLUTININ IN CORD SERUM							
			PRESENT						ABSENT	
			COMPLETE		INCOMPLETE		TOTAL			
			NO.	%	NO.	%	NO.	%	NO.	%
Anti-A	Homospecific	54	39	72.2	11	20.4	50	92.6	4	7.4
	Heterospecific	27	24	88.9	3	11.1	27	100.0	0	0
	All cases	81	63	77.6	14	17.6	77	95.2	4	4.8
Anti-B	Homospecific	76	43	56.6	13	17.1	56	73.7	20	26.3
	Heterospecific	48	34	70.9	8	16.6	42	87.5	6	12.5
	All cases	124	77	62.0	21	17.0	98	79.0	26	21.0

*Incompatible agglutinins excluded.

TABLE IV. REPORTS ON PRESENCE OF INCOMPATIBLE ISOAGGLUTININS IN CORD BLOOD

AUTHOR	YEAR	BLOOD GROUP		NO. OF CASES	METHOD USED*	TYPE OF ANTIBODY†	DISEASE IN INFANT
		MOTHER	INFANT				
Aubert et al.[7]	1945	B	A	1	?	?	Fatal HDN
Mitchell et al.[8]	1949	O	A	3	P, W	I	Fatal HDN
Wiener et al.[4]	1949	O	A	1	A	I	HDN
		O	B	1	A	I	HDN
Witebsky[9]	1949	B	A	1	AB	I	Fatal HDN
Robinson et al.[10]	1951	O	A	3	SA	I	HDN
Bryce et al.[11]	1952	O	A	1	AGT	I	HDN
Phansomboon and Pollack[12]	1952	O	A	2	W	I	Fatal HDN
Crawford et al.[13]	1953	O	A	8	AGT	I	HDN
Zuelzer and Kaplan[14]	1954	O	A	22	AGT	I	HDN
		O	A	1	S	C	HDN
		O	B	6	AGT	I	HDN
Seelen[15]	1954	O	A	2	S	C	Sl. jaundice
			Total	52			
Jakobowicz and Bryce[16]	1948	O	A	3	S	C	None
		A	AB	1	S	C	
Wiener et al.[4]	1949	O	A	4	A	I	
		O	B	1	A	I	
		A	B	1	A	I	
Zuelzer and Kaplan[17]	1954	O	A	3	AGT	I	
		O	A	1	AGT	I	
		O	A	1	S	C	
			Total	15			

*A = Acacia. AB = serum (group AB). AGT = indirect antiglobulin test. S = saline. P = plasma. SA = serum albumin. W = Witebsky neutralization.[18]

†C = Complete. I = incomplete.

DISCUSSION

Incompatible isoagglutinins in an infant with hemolytic disease were first reported in 1945 by Aubert and associates. We have attempted to summarize additional reports from the literature in Table IV as they were available to us. Most of them were case reports or small series, until Zuelzer and Kaplan[14, 17] reported a larger series in 1954. With the exception of 3 cases, the antibodies in the studies listed in the upper part of the table were incomplete; the methods used for their detection are shown in a separate column. The high incidence—7 out of 52—of fatal HDN is remarkable; it probably does not reflect the general clinical course in ABO incompatibility but rather the selected character of the series. Similarly, 19 of 34 cases listed by us in Table I were severe enough to necessitate replacement transfusion.

Table IV also contains data from the literature on presence of incompatible agglutinins in infants without any clinical disease. Significantly, 5 of 15, or one-third, of these antibodies were complete, i.e., demonstrable in saline medium. This incidence is similar to that of our own series (Table II).

On the basis of these findings of previous workers and of our own observations, the following remarks appear to be in order:

1. Incompatible agglutinins are present, as a rule, in the serum of infants affected with HDN caused by ABO incompatibility.

2. The antibodies are, as a rule, of the "incomplete" type, and hence require for their demonstration methods such as the antiglobulin technique or enzyme-treated red cells.

3. In our experience, the antiglobulin test or the technique employing papain-treated red cells proved capable of demonstrating the incompatible agglutinin in each of the 34 cases of hemolytic disease caused by AB incompatibility which we had an opportunity to investigate. Papain-treated red cells were sometimes successful in demonstrating the antibody when the indirect antiglobulin test failed to do so, and vice versa. Both methods should be used in each case. Our experiences with papain-treated cells appeared to confirm the statement by Spielmann[19] that the strongest and most rapid agglutination of group A red cells by anti-A agglutinins was seen with papain-treated red cells.

4. In our series, 14 of 123 infants in heterospecific pregnancies showed incompatible agglutinins *without* evidence of hemolytic disease. In 4, the agglutinins were detectable in saline, and hence presumably a "complete" antibody. Hence, while incompatible agglutinins were found in every case of HDN caused by AB incompatibility, this finding is not specific for the disease because they were also found in about 10 per cent of infants without demonstrable disease. This is not at all surprising since passage of anti-A or anti-B from mother to infant may occur without manifest clinical consequences. On the other hand, because of the frequently mild disease, the findings of incompatible isoagglutinins is to be considered as a warning, inviting closer observation and additional testing of the newborn.

5. Among the 34 infants with HDN caused by AB incompatibility, 27 involved incompatibility to the A antigen, and 7 to B. Several authors have previously recorded and commented on the greater frequency of maternal isosensitization to the A than to the B antigen as cause of HDN.[4-6] In our series of normal infants born from heterospecific pregnancies (Table II), transplacental transfer of incompatible anti-A was observed in 9 of 72 cases, and

of incompatible anti B in 5 of 51 cases These figures do not suggest that the placenta represents a greater barrier to incompatible anti B than to incompatible anti A

As far as transplacental transfer of compatible agglutinins (Tables II and III) is concerned, the following observations were made mothers of group O transmit compatible isoagglutinins (anti A as well as anti B) much more frequently to their infants than do mothers of group A This holds true also for heterospecific pregnancies of O mothers with infants of group A or B Mothers of group B transmit isoagglutinin anti A almost as frequently as O mothers, but the antibody is more frequently incomplete While, in general, mothers transmit anti A more frequently to their infants than anti B, this difference by itself does not seem to be capable of explaining the greater incidence of HDN caused by maternal isosensitization to A than to B As a matter of fact, incomplete anti B antibodies which most likely would be responsible for HDN appeared to traverse the placental barrier with about the same frequency as did incomplete anti A The more frequent appearance of anti A than of anti B in cord bloods may be merely the reflection of the fact that anti A isoagglutinin reach higher titers in the maternal serum than do anti B agglutinins The explanation of the difference in the incidence of HDN caused by A and B isosensitization must be looked for in factors other than differences in transplacental transfer of the corresponding antibodies

Our observations are in good accord with data obtained by other investigators on the frequency of transplacental transfer of isoagglutinins Thus Polayes, Lederer, and Wiener[20] found anti A in approximately 70 per cent and anti B in 53 per cent of cord bloods of infants whose mothers possessed these antibodies Since these antibodies were demonstrated with saline suspended red cells, they may correspond to our category of "complete" antibodies of 77 6 per cent for anti A and 62 0 per cent for anti B (Table III) However, it must be kept in mind that the ability to agglutinate saline suspended red cells by itself cannot be always taken as proving an antibody to be complete One of us (I D[21]) showed that occasional serum specimens with high titers of incomplete Rh antibodies may agglutinate saline suspended red cells when tested in undiluted serum or even in low dilution Recently, Munk-Andersen[22] specifically pointed out that incomplete anti A and anti B agglutinins required even less colloid than Rh antibodies for bringing about agglutination of suitable red cells In the present work the incomplete nature of isoagglutinins was considered as proved only if either saline suspended red cells were not clumped at all or at a titer at least 2 dilutions lower than the titer obtained with the indirect antiglobulin test or with papain treated red cells

Using saline suspended red cells, Tovey[23] found that in homospecific pregnancies anti A was transmitted from mother to infant in 85 per cent and anti-B in 74 per cent

On the basis of quantitative estimations of maternal and neonatal antibodies, he interpreted these findings as primarily reflecting higher maternal titers of anti-A than of anti-B and not indicating differences in placental permeability for the two antibodies. That, in addition to "complete" isoagglutinins, cord bloods may contain "incomplete" anti-A and anti-B was demonstrated by Hirszfeld and associates[24] and by Wolf[25] by means of a dextran method, and by Wiener and Unger[6] who employed acacia or the indirect antiglobulin (Coombs') technique.

SUMMARY

1. Isoagglutinins anti-A or anti-B capable of reacting with red cells of the same ABO group as the infant (incompatible agglutinins) were demonstrated in the blood of each of 34 infants with HDN caused by ABO incompatibility.

2. The incompatible antibodies were of the "incomplete" type and could be best demonstrated with the indirect antiglobulin test or with papain-treated red cells.

3. Incompatible agglutinins were also found in approximately 12 per cent of cord bloods of normal infants born from heterospecific pregnancies; about one-third of these antibodies were of the "complete" type.

4. Data collected from the literature together with our own observations support the usefulness of tests for "incompatible" isoagglutinins in the neonatal diagnosis of HDN caused by ABO incompatibility.

5. From investigations of the transplacental transfer of anti-A and anti-B agglutinins it appears unlikely that differences in placental passage can account for the differences in the incidence of HDN caused by A and B incompatibility, respectively. The commonly higher titer of anti-A in the blood of the mother is probably sufficient to account for the difference.

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A COMPARATIVE STUDY OF THE ASHBY DIFFERENTIAL AGGLUTINATION, CARBON^{14} AND IRON^{59} METHODS FOR THE DETERMINATION OF RED CELL LIFESPAN

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ANEMIA not resulting from extravascular blood loss can only be due to a decrease in the rate of production of red cells, a decrease in the lifespan of the red cell, or a combination of these two factors. With the development in this laboratory of the radioactive iron method for the measurement of the rate of production of red cells, it was shown that in chronic leukemia the red cell production rate is usually normal or increased.[1] This implied that the red cell lifespan might be shortened. Studies of the lifespan of the red cell using C^{14}-labeled glycine showed that in patients with chronic myelogenous leukemia, and in some patients with chronic lymphatic leukemia, the red cell lifespan was shortened.[2] This is a direct method for the measurement of the red cell lifespan. At the time these studies were in progress, it became apparent that a re-evaluation of the Ashby differential agglutination technique for determining the lifespan of the red cell was necessary, since with this method normal cells are transfused to the patient or, on the other hand, red cells from a patient are transfused into a normal recipient.[3] In neither case is the red cell lifespan measured in its normal environment. It is entirely possible that the recipient will not treat the transfused cells in the same manner as his own. It is possible that an individual might produce red cells that are defective and have a short lifespan, while normal cells transfused to him would have a normal lifespan. For this reason, studies of the lifespan of red blood cells were undertaken using simultaneously the Ashby, the C^{14}-labeled glycine, and the Fe^{59} techniques.

METHODS

Thirteen patients were studied (see Appendix). Patients with blood groups A, AB, or B were transfused with red cells from group O normal donors, and the patients with group O and subgroup M red cells were transfused with subgroup N red cells.[3-7] The transfused red cells were counted at least once a week, according to the procedure of Sheets and associates.[4]

Plasma iron clearance and in vivo iron studies were done with Fe^{59}, as previously described.[1, 8] The Fe^{59} red cell uptake curves were followed for at least 150 days, except in patients R. F. and F. W.

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The lifespan of the red blood cells was measured with C^{14} labeled glycine as described previously.[2]

The blood volume was determined with P^{32} labeled red cells.[9]

RESULTS

The blood volumes are presented in Table I. Table II gives the plasma radioiron clearance data and summarizes the Fe^{59} in vivo studies.

TABLE I

PATIENT	BLOOD VOLUME		TOTAL RED CELL VOLUME		PLASMA VOLUME		HEMATOCRIT (PER CENT)	
	ML.	ML./KG	ML.	ML./KG	ML.	ML./KG	PRCV*	PCV*
1 J. J.†								
2 J. S.	4,846	101.0	1,212	25.3	3,198	66.6	25	34
3 M. B.	3,671	61.2	1,597	26.6	1,909	31.8	44	47
4 F. T.	7,135	103.3	1,712	24.8	3,924	56.8	24	45
5 T. W.	3,558	61.1	1,281	22.0	2,277	39.1	36	
6 F. W.	4,419	53.7	1,282	15.6	3,137	38.1	29	
7 R. M.	4,260	67.0	1,747	27.5	2,513	39.5	41	
8 R. G.	5,040	67.5	2,117	28.3	2,873	38.5	42	
9 T. B.	5,391	77.9	2,208	30.8	2,963	47.1	40	43
10 R. F.	2,820	58.8	1,128	23.1	7,692	35.7	40	
11 A. F.	5,854	80.5	2,019	27.8	3,835	52.8	34	
12 L. F.	3,179	69.9	1,717	37.7	1,462	32.2	54	
13 T. A.	5,151	76.6	1,753	26.0	3,404	50.6	34	

*In cases in which the W.B.C. was elevated, both the packed red cell volume (PRCV) and the packed cell (red and white) volume (PCV) are indicated.

†No blood volume data available for this patient.

TABLE II

PATIENT	DATE	FE TURNOVER FOR HGB FORMATION (MG./KG./DAY)	IN VIVO FE^{59} STUDIES
1 J. J.*			
2 J. S.	2/15/52	1.69	Myeloid metaplasia
3 M. B.	2/8/54	0.17	Normal
4 F. T.	9/25/52	0.25	Splenectomy
5 T. W.	5/14/53	0.51	Normal
6 F. W.	4/22/53	0.11 0.14	High liver uptake†
7 R. M.	2/25/53	0.25	High liver uptake†
8 R. G.	5/6/52	0.59	Persistent spleen curve
9 T. B.	1/26/54	0.19	Persistent spleen curve
10 R. F.	4/3/52	0.30	Normal
11 A. F.	7/1/54	1.39	Secondary spleen curve
12 L. F.	11/10/52	0.57	(Not done)
13 T. A.*			

*Fe^{59} studies not carried out.

†Liver uptake: F. W. 2.77 mg. Fe per gram of tissue; R. M. 8 mg. Fe per gram of tissue measured from liver biopsies.

Ashby Studies.—When the rate of decrease of transfused cells was predominantly linear, the linear segment of the data was fitted to a straight line by the least squares method. The red cell lifespan and the maximum concentration of inagglutinable cells were obtained by extrapolation (see Figs. 1 and 2). In the 10 patients in this group the red cell lifespan varied from 98 to 136 days.

The other 3 patients, A. F. (Fig. 3), L. F. (Fig. 4), and T. A. (Fig. 5), showed an exponential rate of disappearance of donor cells (Table III). The

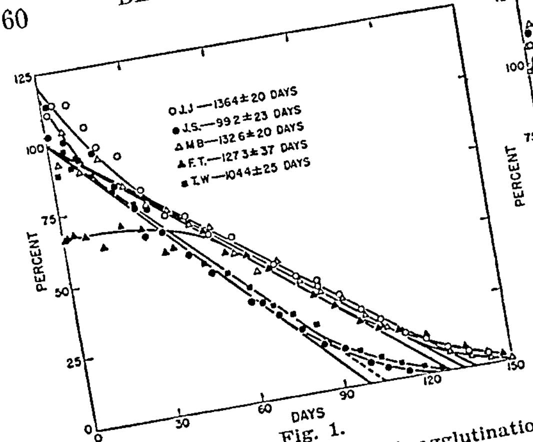

Fig. 1.

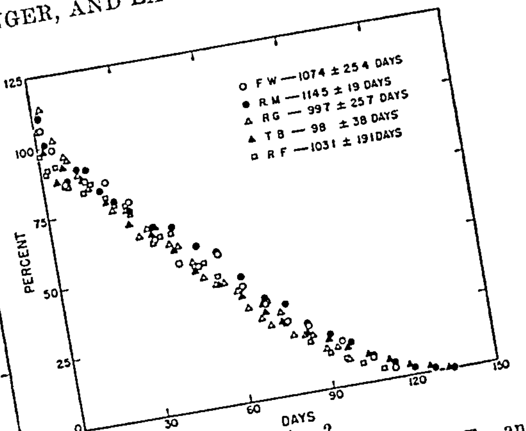

Fig. 2.

Fig. 1.—Ashby differential agglutination data for Patients J.J., J.S., M.B., F.T., and T.W.

Fig. 2.—Ashby differential agglutination data for Patients F.W., R.M., R.G., T.B., and R.F.

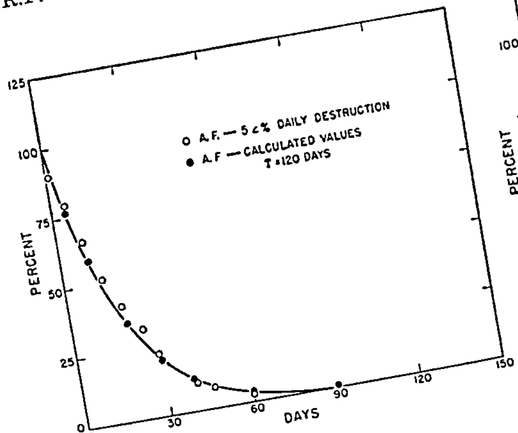

Fig. 3.

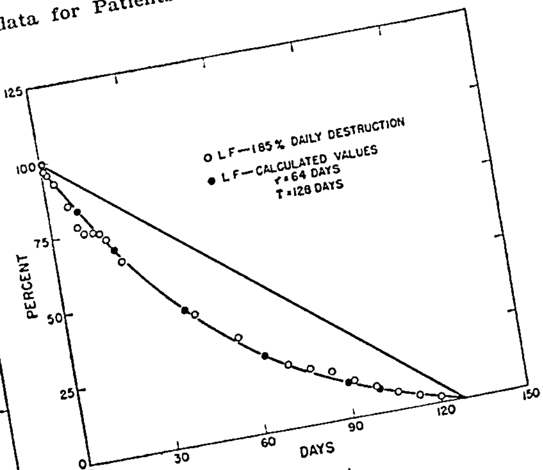

Fig. 4.

Fig. 3.—Ashby differential agglutination data for Patient A.F.

Fig. 4.—Ashby differential agglutination data for Patient L.F.

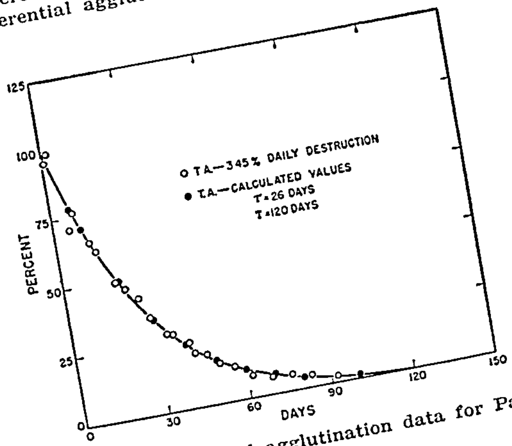

Fig. 5.—Ashby differential agglutination data for Patient T.A.

theoretical values were calculated according to Sheets and associates,[4] assuming a random destruction at a constant rate plus normal aging

$$N = N_0 \left[1 - \frac{t}{T}\right] e^{-\frac{0\ 693\ t}{\tau}}$$

where N = Concentration of donor cells at time *t* after transfusion, N_0 = Concentration of donor cells at *t* = 0, T = Time after transfusion when all the donor's cells would have been lost by senescence, and τ = Time after transfusion when *N* is half the number of cells which would have been present if loss were due only to senescence

That the exponential disappearance shown by these 3 patients can be explained as a combination of random destruction and normal aging is shown by the close agreement of the observed values with those calculated from this equation [4]

TABLE III

PATIENT	SEX	DISEASE	RED CELL LIFESPAN (DAYS)			RATE OF RED CELL FORMATION (IN % RENEWAL/DAY FROM FE^{59} STUDIES)
			ASHBY	C^{14}	FE^{59}	
1 J J (Figs 1, 9)	M	Chronic myelogenous leukemia (?) and tuberculosis	136 4	142	142	
2 J S (Figs 1, 6)	F	Myeloid metaplasia	99 2	115		6 7
3 M B (Figs 1, 7)	F	Chronic myelogenous leukemia	132 6	114		0 67
4 F T (Figs 1, 8)	M	Chronic myelogenous leukemia	127 3	118	120	1 01
5 T W (Figs 1, 16)	M	Anemia of undetermined etiology	104 4	76	63	2 31
6 F W (Figs 2, 14)	M	Aplastic anemia, hemochromatosis	107 4	87	80	0 71
7 R M (Figs 2, 15)	M	Diabetes mellitus, hemochromatosis	114 5	113		0 91
8 R G (Figs 2, 12)	M	Giant follicle lymphoblastoma	99 7	105	99	2 08
9 T B (Figs 2, 10)	M	Chronic lymphatic leukemia	98 0	101	88	0 62
10 R F (Figs 2, 13)	F	Giant follicle lymphoblastoma	103 1	97		1 30
11 A F (Figs 3, 17)	M	*Chronic lymphatic* leukemia	*5 2% daily* destruction	31		5 7
12 L F (Figs 4, 11)	F	Secondary polycythemia	1 85% daily destruction	76	80	1 51
13 T A (Fig 5)	M	Chronic lymphatic leukemia	3 45% daily destruction			

One patient (F T) with chronic myelocytic leukemia (Fig 1) showed no destruction for at least 40 days This patient had had a splenectomy one year earlier and had been given a therapeutic dose of 4 mc of P^{32} intravenously two days after the transfusion of Ashby cells By reducing the rate of production of red cells and also the blood volume, P^{32} probably effected a temporary net concentration of donor cells

The variation from a straight line in the disappearance of transfused cells in the early days following the transfusion has been attributed to the difference between the rate of breakdown of the donor's and recipient's red cells.[5] For this reason, the same donor was used several times for several recipients.

TABLE IV

DONOR	RECIPIENT	AGING TIME (DAYS)	RED CELL LIFE (DAYS)	INITIAL VALUE (PER CENT)	CALCULATED RED CELL LIFE DONOR (DAYS)
R. W	T. A.		R. D.		
	T. W.	79.4	104.4	100	104.4
	M. B	112.6	132.6	100	132.6
T. M.	F. W.	82.0	107.4	110	118.1
	J. J.	116.4	136.4	120	163.7
F. G.	R. F.	84.0	103.1	92	94.9
	L. F.		R. D.		

R. D. = random destruction.

The results are tabulated in Table IV. The red cell life of the donor cells may be calculated in the following way[5]:

$$\text{Red cell life donor} = N_o\ (k),$$

$$\text{where } k = \frac{1}{\text{slope (straight segment of Ashby curve)}}\ .$$

For donor R. W. (Table IV) there was no such initial variation. The red cell life of the donor then should be the same as that of the recipient, but the 2 recipients had different red cell lifespans. With the donor T. M. there was a different initial destruction rate in the two recipients, and the lives of the same donor cells calculated on this basis are different. It is possible then that the variation from a straight line in the first part of the curve is due principally to the fragility of the cells toward transfusion manipulations.

Evans and co-workers[5] state that the deviation from linearity in the last part of the Ashby differential agglutination curves can be explained by assuming that normal cells "undergo a period of aging" and that "aged cells are broken down by a random, first-order process." Table IV shows that the "aging times" of cells from the same donor vary in different recipients.

TABLE V. COMPARISON OF DURATION OF TAIL PORTION OF ASHBY CURVE WITH C^{14} CURVE

PATIENT	ASHBY (DAYS)	C^{14} (DAYS)
1. J. J.	± 20	± 20
2. J. S.	± 23	± 23
3. M. B.	± 20	± 19
4. F. T.	± 37	± 43
6. F. W.	± 25	± 23
7. R. M.	± 19	± 35
8. R. G.	± 26	± 29
9. T. B.	± 38	± 37
10. R. F.	± 19	± 18

The tail portion of the curve calculated according to Evans and associates[5] shows an average life of the worn cells ranging between ±19 to ±38 days, in agreement with that found by Evans and co-workers (Table V).

C^{14} and Fe^{59} Labeled Cell Method.—Ten of the 12 patients studied showed a plateau of Fe^{59} or C^{14} specific activity (Figs. 6-10, 12-16). The mean of the plateau was considered as 100 per cent.

In patients L. F. and A. F. (Figs. 11 and 17), whose graphs showed no plateaus, the highest values were considered as 100 per cent. In these two patients the red cell lifespan as measured with carbon^{14} labeled glycine represents an upper limit. It is quite likely the true mean red cell lifespan is shorter. The effect of continual synthesis of labeled hemoglobin from persisting labeled glycine tends to make the slope of the curve of hemoglobin carbon^{14} content smaller than would occur if there were not a continual synthesis. It is relatively easy to show that when the red cell lifespan is finite and normal this tends to overestimate the red cell lifespan only slightly.[10] Mathematical methods for the correction of the observed carbon^{14} content of hemoglobin when the pattern is one of a very short red cell lifespan and random destruction, have not been devised. It can only be stated that the actual red cell lifespan will be less than that calculated from the data and, at present, of an unknown magnitude.

The red cell lifespans as determined by C^{14} and Fe^{59} are given in Table III. They are obtained by taking the time between the ascending and descending parts of the curves at half the distance between the upper and lower plateaus. The descending part of the curve in the C^{14} results, which corresponds to the random destruction of worn cells, might be compared with the exponential destruction of the worn Ashby cells (Table IV). Six patients (T. W., F. W., T. B., A. F., L. F., and T. A.) had short red cell lifespans as determined either by the Ashby technique or by both the Ashby and C^{14} methods. Of the 10 patients on whom in vivo Fe^{59} studies were done (see Table II), 4 had abnormal spleen curves. In 3 there was either a rise in the counting rate over the spleen followed by a fall and then a secondary rise, or there was a rise with no fall. These types of spleen curves have been interpreted as indicating premature destruction of red cells in the spleen.[11] Because of the large reutilization of iron, the curves obtained using the Fe^{59} method do not have the sharp fall associated with the C^{14} or N^{15} studies, and analysis of the lifespan is not as satisfactory. Since it cannot be assumed that any of these patients was in a steady state with regard to red cell production and destruction, calculation of the red cell lifespan from the red cell iron turnover is misleading.

Comparative Study of the Red Cell Lifespan With the Three Methods.—For comparison, the Ashby, C^{14}, and Fe^{59} results were plotted on the same graph. The Ashby data were corrected for daily destruction in the following manner. If the red cell life is T, at N days after the beginning of the experiment, the plotted inagglutinable cells are equal to

$$\frac{100\ (T-N)}{(\%\ \text{inagglutinable cells at } N)\ T}$$

When the red cells produced in the patient are normal and destroyed only through senescense, the results should agree in all three methods. They agree in 9 patients within the limits of error (Table III), and when the rate of production is sufficient, the total red cell volume will be normal.

Patient T. W. (Fig. 16) showed a normal red cell life with the Ashby technique and a finite but shortened red cell life with C^{14} and Fe^{59}. Here the

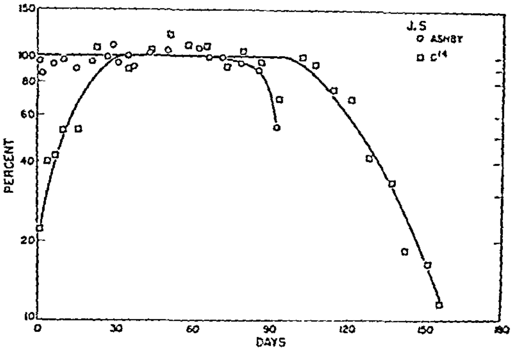

Fig. 6.

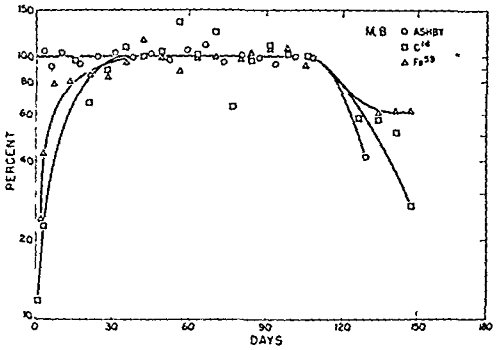

Fig. 7.

Fig. 6.—Red cell lifespan curves by the Ashby and C^{14} methods for Patient J.S.
Fig. 7.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient M.B.

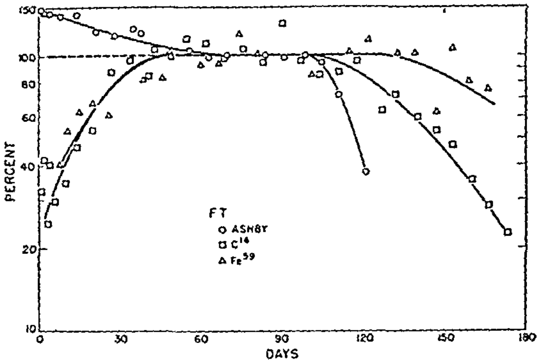

Fig. 8.

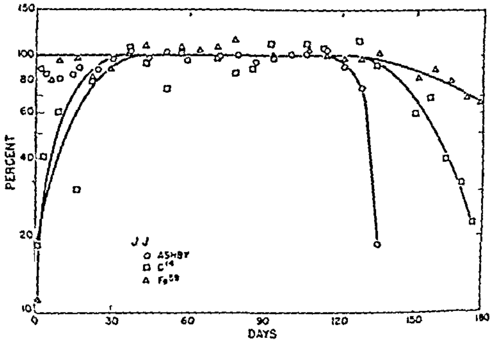

Fig. 9.

Fig. 8.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient F.T.
Fig. 9.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient J.J.

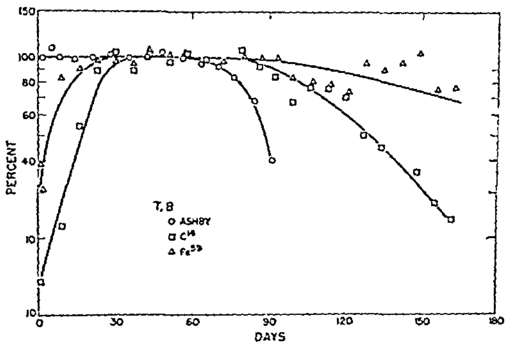

Fig. 10.

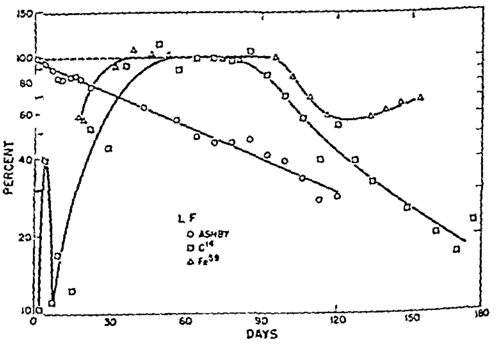

Fig. 11

Fig. 10.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient T.B.
Fig. 11.—Red cell lifespan curves by the Ashby, C^{14}, and Fe^{59} methods for Patient L.F.

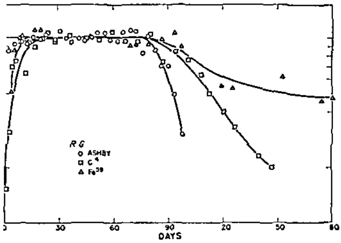

Fig 12

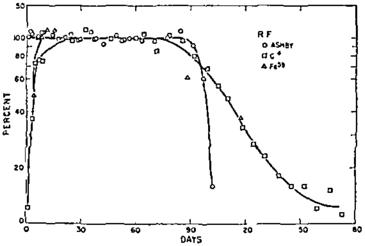

Fig 13

Fig 12 —Red cell lifespan curves by the Ashby C^{14}, and Fe^{59} methods for Patient R G
Fig 13 —Red cell lifespan curves by the Ashby C^{14} and Fe^{59} methods for Patient R F

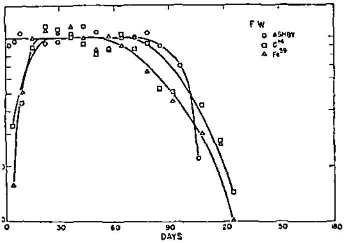

Fig 14

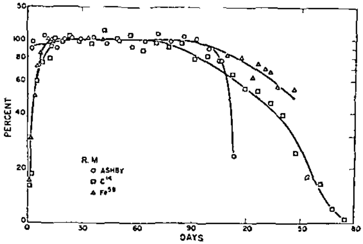

Fig 15

Fig 14 —Red cell lifespan curves by the Ashby C^{14} and Fe^{59} methods for Patient F W
Fig 15 —Red cell lifespan curves by the Ashby C^{14} and Fe^{59} methods for Patient R M

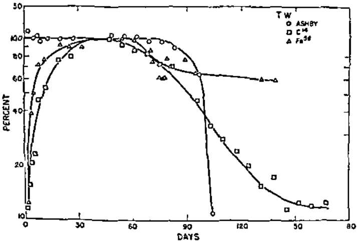

Fig 16

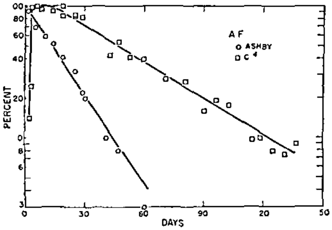

Fig 17

Fig 16 —Red cell lifespan curves by the Ashby C^{14} and Fe^{59} methods for Patient T W
Fig 17 —Red cell lifespan curves by the Ashby and C^{14} methods for Patient A F

quality of the cells must have been deficient, as normal cells survive normally in the patient while the patient's own cells have a finite but shortened lifespan. The rate of production as measured with Fe^{59} was elevated, indicating an increased production of red cells which partially compensated for their short lifespan, although the patient was anemic by blood volume standards.

Patient L. F. (Fig. 11) showed random destruction with a rate of 1.85 per cent per day (normal, 0.85 per cent) with the Ashby technique and a short but apparently finite red cell life of 76 days for C^{14} and 80 days for Fe^{59}. The patient's cells might have had a normal red cell life if given to a normal subject, but in the patient they undergo a random destruction as shown by the Ashby technique. The high plasma iron turnover associated with red cell formation indicates an increased production rate that compensates for the increased destruction and keeps the total red cell volume at a slightly polycythemic level.

In Patient F. W. (Fig. 14) the red cell life obtained by the Ashby technique was normal (107 days) and somewhat longer than the red cell life as measured by the two other methods (87 days for C^{14} and 80 days for Fe^{59}). The rate of formation of red cells as measured by Fe^{59} was low, indicating a decreased production of red cells which might have been slightly deficient in some as yet unknown manner as compared to normal red cells. Both these factors would explain the anemia of this patient, who needed frequent transfusions.

Patient F. T. (Fig. 8), who had been splenectomized, had a very long red cell life by all three methods, and all three methods yielded comparable values.

Red Cell Lifespan in Disease.—

Chronic myelogenous leukemia: In previous studies it was reported that the red cell lifespan was shortened in chronic myelogenous leukemia but that the lifespan was finite.[2] Two patients with chronic myelogenous leukemia in this study had normal red cell lifespans. In one (F. T.) this was following a splenectomy and is in agreement with Ragnar Berlin's[12] observations on the lengthening of the red cell lifespan in chronic myelogenous leukemia following splenectomy. In the other patient (M. B.) this was very early in the course of her disease, presumably within 6 months of the onset of leukemia. Further observations at a later time would be of interest to determine if and when shortening of red cell lifespan does develop.

Chronic lymphatic leukemia: In agreement with previous studies, the lifespan of the red cell in chronic lymphatic leukemia was found to be either normal or very short with a pattern of random destruction.[2] In patient A. F. conversion to a normal red cell lifespan was observed following splenectomy.

Giant follicle lymphoblastoma: Both patients with giant follicle lymphoblastoma (R. G. and R. F.) had a normal red cell lifespan.

Unfortunately these studies do not differentiate in those patients with a short red cell lifespan—either with random destruction or with a shortened but finite red cell lifespan—the nature of the defect leading to a decrease in the lifespan of the cell. In Patient A. F. the conversion from a short red cell lifespan with random destruction to a normal cell life following a splenectomy strongly suggests the presence of an extrinsic factor arising in or mediated through the spleen.[13] In fact, on theoretical grounds, in the absence of hemorrhage, the pattern of random destruction is best explained by some extracorpuscular factor, and the shortened but finite red cell lifespan by some intracorpuscular defect These studies are not conclusive in this regard, however

CONCLUSIONS

1 In 8 patients the red cell lifespan was normal as determined by glycine labeled with C^{14} and by the Fe^{59} and Ashby techniques The results of all three methods were in good agreement

2 The Ashby technique is of value in interpreting the data shown by the C^{14} and Fe^{59} methods

3 In the 2 cases presumably with hemolysis due to extracorpuscular factors all three methods showed a very short cell life

4 In 2 cases (T W and F W) the quality of the cells produced was defective, and the Ashby technique showed a normal red cell life while the C^{14} and Fe^{59} methods showed a shorter red cell life

APPENDIX

Case 1—J J, a 65 year old white man, was first seen here in September, 1953 A diagnosis of pulmonary tuberculosis was made in 1951, and the patient was treated In November, 1952, elevation of the white cell count and an abnormal differential suggested chronic myelogenous leukemia The patient was placed on Myleran in 1954, and the white count fell to normal with a normal differential In December, 1954, a lobectomy was performed because of cavitation in the left upper lobe The patient expired 12 hours after surgery At autopsy no evidence of myelogenous leukemia could be found Whether this should be viewed as a leukemoid response to tuberculosis or whether it represented an excellent response to Myleran with a conversion of the bone marrow to normal is problematical The absence of histologic evidence of leukemia in spleen, liver, and bone marrow made the diagnosis difficult

Case 2—J S, a 51 year old white woman, was first seen in February, 1952 She gave a history of an onset of arthritis in 1949 In September, 1950, the patient was told a blood count showed leukemia In 1951 the spleen became palpable Physical examination disclosed a thin, fairly developed woman, with deforming rheumatoid arthritis of the elbows, wrists, fingers, knees, and ankles The blood count showed R B C 3 53 million, hemoglobin 7 8 Gm , platelets 400,000, and W B C 140,000, with a differential compatible with that of myelogenous leukemia Iron studies showed an excessive iron turnover, only part of which could be attributed to excessive turnover through the red cells The serial spleen site counting rate curve indicated a primary path through the spleen, with a subnormal rise in the marrow site counting rate, suggesting splenic extramedullary erythrogenesis The decline in the counting rate over the spleen simultaneously with the ascending radioactivity in the red cells is believed to be confirmatory of splenic erythropoiesis As a result of liver, spleen, and bone marrow biopsies and the radioiron studies, a diagnosis of myeloid metaplasia was made The patient has been treated throughout with cortisone

Case 3—M B, a 38 year old white woman, was first seen here in February, 1954, with chronic myelogenous leukemia of approximately 4 months' duration At the time these studies were carried out, the liver and spleen were not palpable The blood count was 4 63 million, with 14 6 Gm of hemoglobin, platelets 530,000, and white count 81,500, with a differential compatible with that of chronic myelogenous leukemia This is the first patient with chronic myelogenous leukemia studied in this laboratory to have a normal red cell lifespan in the presence of a spleen (see Case 4 F T, for results in splenectomy) It is probable that this woman was studied so early in the course of the disease that alteration in the lifespan of the red cell was not yet evident

Case 4—F T, a 36 year old white man, was first seen here in May, 1952, with chronic myelogenous leukemia of one year's duration A splenectomy had been done in 1951, fol lowed by x-ray therapy. At the time he was first seen here the blood count was 3.58 million; hemoglobin 8.5 Gm.; platelets 1,880,000, and W.B.C. 331,000.

CASE 5.—T. W., a 67-year-old white man, was first seen here in January, 1953, with the principal complaint of weakness and fatigue for the preceding 18 months. He was referred here with a diagnosis of anemia of unknown etiology. At the time of his first visit the blood count showed R.B.C. 3.94 million; hemoglobin 11.8 Gm.; and W.B.C. 5,200 with a normal differential. The blood volume determination showed 22 ml. per kilogram of red cells and a plasma volume of 39.1 ml. per kilogram. This was a moderate anemia, but despite extensive investigation the cause has not yet been established.

CASE 6.—F. W., a 74-year-old white man, was seen at this laboratory in March, 1953, with a history of anemia. A diagnosis of carcinoma of the prostate was made following a transurethral resection in 1952. Following surgery, the patient was found to be anemic and did not respond to therapy. Radioactive iron studies showed that this anemia was due to a failure in red cell production. This man required frequent transfusions to maintain the blood count at approximately 2.5 to 3.5 million, with 6.8 to 9.3 Gm. of hemoglobin. The white cell count was never remarkable. Biopsy of the bone marrow showed hypoplasia. The liver biopsy showed hemosiderin pigmentation characteristic of hemochromatosis, probably secondary to multiple transfusions.

CASE 7.—R. M., a 45-year-old white man, was first seen here in October, 1952, with a diagnosis of hemochromatosis and diabetes. At the time these studies were done, the patient had the following blood count: R.B.C. 4.92 million; hemoglobin 13.8 Gm.; and W.B.C. 6,200, with a differential showing 39 per cent segmented and 2 per cent nonsegmented cells, 2 per cent eosinophils, 51 per cent lymphocytes, and 5 per cent monocytes. In vivo iron studies showed a greater than normal amount of iron disappearing from the plasma to liver. A sample of liver tissue contained 8 mg. of iron per gram of liver tissue. Urinalysis showed 3+ sugar. Pigmentation was found on the backs of the arms, legs, periumbilical area, and the axilla. The liver was hard and easily felt almost 2 cm. above the umbilicus. The spleen was not palpable.

CASE 8.—R. G., a 52-year-old white man, was first seen in October, 1949. The patient had noticed right cervical lymphadenopathy in 1944, and a biopsy revealed giant follicle lymphoblastoma. He was treated with x-radiation to the right inguinal and right axillary regions at approximately 6- to 12-month intervals. The last x-ray therapy before being seen here was given a month previously. On physical examination it was noted that there were ½ to 1 cm. nodes in both axillae and in the right inguinal region. No cervical nodes were palpable. The liver was palpable to the level of the iliac crest. The blood count showed R.B.C. 3.61 million; hemoglobin 11 Gm.; and W.B.C. 7,500, with a normal differential. The red cell lifespan studies were started in May, 1952. At this time there was only one node palpable, in the right posterior cervical triangle. The liver and spleen were not palpable. The blood count then showed R.B.C. 4.09 million; hemoglobin 11.3 Gm.; and W.B.C. 6,150, with 45 per cent lymphocytes.

CASE 9.—T. B., a 66-year-old white man, was first seen here in June, 1952, with a known chronic lymphatic leukemia of 10 years' duration. At the time these studies were done the spleen was palpable 2 cm. below the costal margin, the liver edge was palpable about 1 to 2 cm. on deep inspiration, and there was one large (2 to 3 cm.) node in the right axilla with smaller lymph nodes palpable in the left axilla and inguinal regions. The blood count done at this time showed: R.B.C. 3.82 million; hemoglobin 12.8 Gm.; platelets 140,000; and W.B.C. 97,000, with 99 per cent lymphocytes and 1 per cent polymorphonuclear leukocytes.

CASE 10.—R. F., a 26-year-old white woman, was first seen here in March, 1952, at which time the patient gave a history of a mass in the left breast of approximately one month's duration. On biopsy, this was found to be a giant follicle lymphoblastoma. There was also a broad tender mass in the upper abdomen which biopsy showed to be a giant follicle lymphoblastoma. The spleen was enlarged. The blood count at this time was as follows: R.B.C. 3.94 million; hemoglobin 11.7 Gm.; and W.B.C. 10,800, with 49 per cent lymphocytes.

CASE 11.—A. F., a 53-year-old white man, had chronic lymphatic leukemia of 2 years' duration. Because of the fact that he had had several episodes of chills and fever which responded to antibiotic therapy and because of the fact that he had a large palpable spleen with a relatively normal white cell count at the time of his first visit here in the spring of 1954, a biopsy of the spleen was done, as well as a sternal marrow biopsy The spleen tissue showed small, granulomatous lesions, the etiology of which is still unknown despite *extensive serologic and bacteriologic investigations* A *splenectomy was done, following* which a normal red cell lifespan was observed, as measured with C^{14}

CASE 12 —L F, a 47 year old woman, was referred to us in October, 1952, with a *diagnosis of pulmonary fibrosis and secondary polycythemia* The patient had a history of shortness of breath for 10 years, and the pulmonary fibrosis was discovered during an episode of influenza in 1949 An elevated red blood cell count had been found 2 months before her first visit here, and a 500 ml phlebotomy had been performed The patient reported a 30 pound weight loss in the preceding 3 years On physical examination, redness of the face hands, and mucous membranes was noted Decreased resonance of the right middle lobe of the lungs was noted, with increased resonance elsewhere There was an increased heart rate, and the blood pressure was 170/120 RAR * The spleen and liver were not palpable The initial blood count was R B C 7 16, hemoglobin 19 0 Gm , and W B C 15,500, with a normal differential count Blood oxygen saturation was 75 per cent The turnover of iron for red cell *production was increased*

CASE 13 —T A , a 53 year old white man, had chronic lymphatic leukemia He was first seen here in November, 1949 Onset of the disease occurred in the summer of 1949, when enlargement of the lymph nodes was observed Biopsy showed lymphoid hyperplasia suggestive of leukemia His first blood count here showed R B C 3 78 million, hemoglobin 13 Gm , platelets 260,000, and W B C 12,100, with a normal differential A bone marrow puncture done at that time showed 46 2 per cent to be of the lymphocytic series The studies of red cell life were done in 1952 when the patient had a red count of 3 million and a differential white count varying from 44 to 72 per cent lymphocytes The spleen was enlarged to the umbilicus The liver was not palpable From 1949 to this time the patient had received therapy with x ray and P^{32}

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*RAR = right arm recumbent.

STUDIES OF PLATELET SURVIVAL BY TAGGING IN VIVO WITH P^{32}

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INTRODUCTION

WITH isotopic techniques it has become possible to study the survival of normal platelets in normal subjects. Two basic methods have been used for platelet tagging: in one the platelets are tagged in vitro by incubation with the isotope; and in the other the isotope is injected into the subject whose platelets are then tagged in vivo. With the in vitro method, considerable incubation, manipulation, and washing of the platelets are involved and their survival has usually been short, sometimes only a few hours.[1-7] Where the in vivo method of tagging has been employed, the survival in general has been longer.[3, 8, 9] However, in all but a few[8, 9] of the reported experiments, the platelets have been separated and washed at least one time before transfusion. There is considerable evidence that sedimenting of platelets by centrifugation may lead to damage which may result in shortened survival.

Leeksma and Cohen[9, 10] have reported a method of tagging platelets in vivo which avoids platelet manipulation and transfusion. The platelets are tagged by an intramuscular injection of diisopropylfluorophosphonate containing P^{32} (DFP^{32}). Several objections to this method may be raised. First, the DFP^{32} may form a depot in the recipient animal, and tagging may continue over a period of several days rather than for several hours, as is assumed. This would give a falsely long survival. Second, this method when applied to white blood cells gives a survival of only a few hours, whereas with most other techniques the white cells survive for many days or weeks. This suggests the possibility of elution of the absorbed DFP^{32}. Third, there is no proof that the absorbed DFP^{32} does not alter the platelets and therefore alter their survival.

We have developed a technique of tagging platelets in vivo and transfusing them with a minimum of manipulation and no incubation, centrifugation, or washing. Since a transfusion is involved, this method can be used to study the survival of isogenous platelets only; it cannot be applied to the survival of autogenous platelets. However, it has the advantage that the P^{32} is actually part of the platelet structure rather than adsorbed onto the platelet surface or taken up by dynamic metabolic interchange with the plasma phosphates.

METHODS

In brief, the method involves the injection of a donor with P^{32}, waiting an appropriate time while a population of radioactive platelets is produced, and then transfusing the donor's whole blood into a recipient. After the transfusion, samples of platelets are isolated from

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the recipient's blood and their radioactivity is measured. This is repeated at appropriate intervals. The diminishing level of radioactivity in the isolated platelets reflects the disappearance of the transfused platelets.

In the animal work, normal dogs weighing approximately 12 Kg. were used as platelet donors and platelet recipients. The donor animals received 1 mc. of P^{32} intravenously. In the human work, patients with polycythemia vera were the donors and normal volunteers were the recipients. The human donors received 3 to 5 mc. of P^{32} intravenously. One week after the P^{32} injection, blood was drawn into plastic bags with disodium ethylenediaminetetraacetate (EDTA) as the anticoagulant. The volume taken was 500 ml from the humans and 300 ml. from the dogs In the animal work, the recipient dog was prepared by a phlebotomy of 300 ml. This was immediately followed by transfusion with the radioactive blood. At no time in either the human or animal experiments did the interval between the drawing of the radioactive blood and its transfusion exceed 30 minutes

Platelet survival was determined by drawing samples of blood from the recipients at various intervals A two syringe technique was used, all glassware was siliconized, and EDTA was used as the anticoagulant. The platelets were separated by differential centrifugation, and the number of platelets was counted by phase microscopy.[11] The platelets were washed repeatedly and were plated onto an aluminum planchet. The radioactivity was counted by means of a thin-window Geiger tube. To overcome the problem of physical decay of P^{32}, all radioactive samples from each experiment were counted at the same time.

Platelet-free plasma was obtained from samples of whole blood drawn by the two-syringe technique with oxalate as the anticoagulant. The blood was centrifuged at 3,000 r p m. for 2 hours, and a volume of the supernatant plasma was plated onto a planchet and counted in the same manner as the platelets.

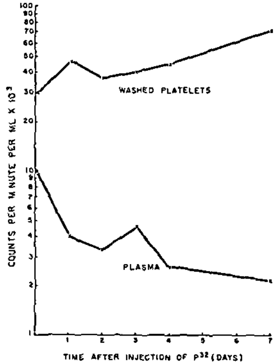

Fig 1 —The incorporation of injected P^{32} into the circulating platelets of a patient with polycythemia vera (Subject A)

RESULTS AND DISCUSSION

Immediately after the injection of P^{32}, the radioactivity of the plasma is high (Fig 1), but it rapidly falls to a low level and thereafter gradually disappears During this disappearance the radioactivity of the platelets climbs to a level which is many times that of the ambient plasma, then gradually subsides At this peak of activity the platelets are harvested

The activity which appears in the platelets separated from the first sample (drawn 30 minutes after injection) may represent P^{32} adsorbed onto the surface of the platelet. Most of the injected P^{32} probably enters the phosphorus pool of the body, and megakaryocytes draw upon this material for the phosphorus that is used in the formation of new platelets. The appearance of new platelets containing P^{32} in their stroma probably accounts for the rising platelet activity during the first 7 days, in the face of a falling plasma activity. The ideal time for phlebotomy appears to be 7 days after the injection, since the platelet activity at that time is at its highest point and plasma activity is low.

A second example of plasma and platelet radioactivity in a human polycythemic subject after injection of P^{32} is shown in Fig. 2. Here the ideal time for phlebotomy would have been the sixth day.

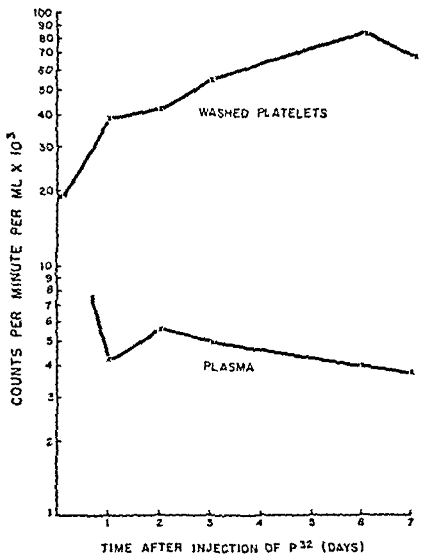

Fig. 2.

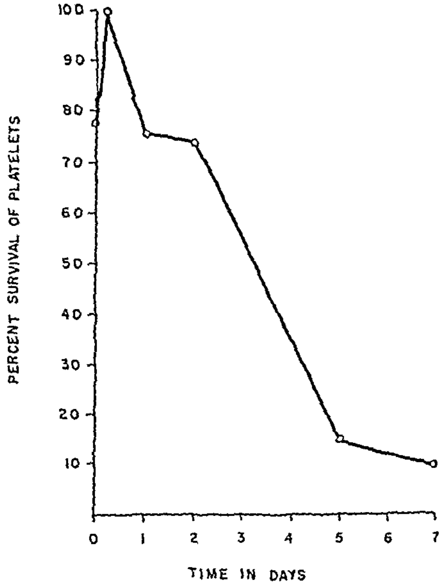

Fig. 3.

Fig. 2.—The incorporation of injected P^{32} into the circulating platelets of a patient with polycythemia vera (Subject B).

Fig. 3.—The survival of transfused radioactive platelets in recipient Dog 1.

Fig. 3 shows the typical survival of radioactive platelets in a normal dog recipient. The curve of decay appears to be exponential, with a half-life of about 3 days. It reached the 10 per cent level on the seventh day. On the day of the transfusion the 30-minute sample showed much less activity than the 3-hour sample. This rise in platelet radioactivity during the first hour post transfusion was a regular occurrence. It may have been due to transient sequestration of the transfused platelets. An alternative explanation is that P^{32} from the donor's plasma was transferred to the recipient's platelets.

To test this possibility, a dog was given intravenously 1 mc. of P^{32}. One week later the animal was phlebotomized. Platelet-free plasma was obtained from the blood and transfused into a recipient dog. Thereafter no significant radioactivity could be demonstrated on the recipient's platelets, which indicated that there was no significant transfer of P^{32} from transfused plasma to the recipient's platelets.

It seems more likely, therefore, that the rise in platelet radioactivity after the 30 minute sample was because of the appearance of platelets which had been briefly sequestrated following their transfusion

To prove that the curves we obtain are truly a measure of platelet survival and not due to artefact or to P^{3} reutilization, an experiment was carried out in which nonviable platelets were transfused A donor animal received

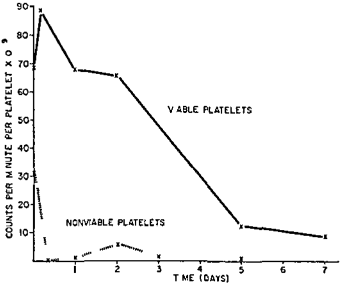

Fig 4 —The survival of transfused radioactive platelets The curve representing loss of viable platelets is the same as Fig 3 shown for comparison The nonviable platelets had been stored in glass

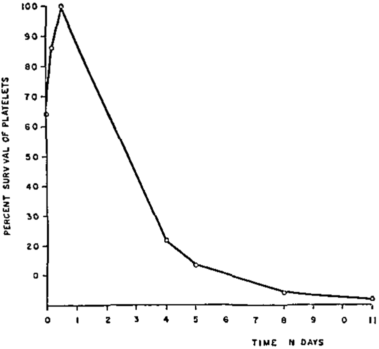

Fig 5 —The survival of transfused radioactive platelets in recipient Dog 2

1 mc of P^{32} and 1 week later was phlebotomized However, instead of carrying out the phlebotomy with plastic bags and EDTA as the anticoagulant, routine blood bank equipment was used (glass vacuum bottles with ACD solution) The blood was kept at 4° C for 24 hours, then transfused It is well known that such treatment results in nonviable platelets When platelet activity in this recipient was measured, only the sample drawn 30 minutes after transfusion showed significant activity Apparently some platelets survived for these few minutes, but by the fifth hour post transfusion these platelets had

disappeared. In Fig. 4, there is a comparison of platelet survival in this dog with platelet survival in Dog 1 shown in Fig 3.

Fig. 5 shows the survival of radioactive platelets in a second dog transfused with blood obtained by phlebotomy into a plastic bag. Platelet sequestration was even more marked and lasted longer in this animal than in Dog 1 (Fig. 3). In Dog 2, the peak in platelet activity did not occur until the end of day 1. The curve again appears to be exponential, this time with a 2½ day half-life and reaching the 10 per cent level on day 6. Some platelet activity was still present on day 11. It is unlikely that the radioactivity in platelets which persists so long after the transfusion could be due to adsorption of P^{32} from the plasma, because plasma activity in the recipient approaches background levels by the second or third posttransfusion day. An alternative explanation is that the residual activity is due to reutilization of phosphorus from platelets, red cells, and plasma. However, this source of phosphorus is so small in comparison with the total body phosphorus pool that the explanation seems unlikely. Furthermore, if reutilization were a significant problem, it would have evidenced itself in the dog that received nonviable platelets (Fig. 4). We believe this residual activity is because of a few transfused platelets that actually survive a long time. Perhaps these are the very youngest platelets in the transfused blood.

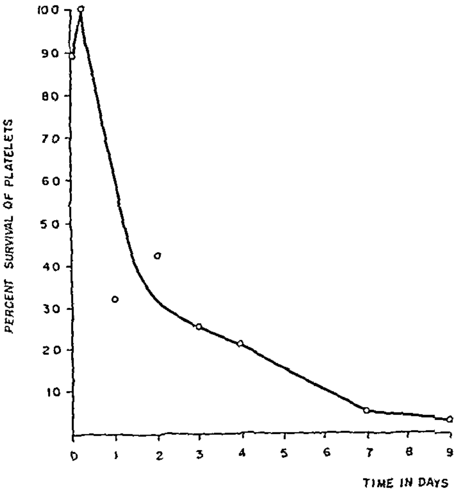

Fig. 6.

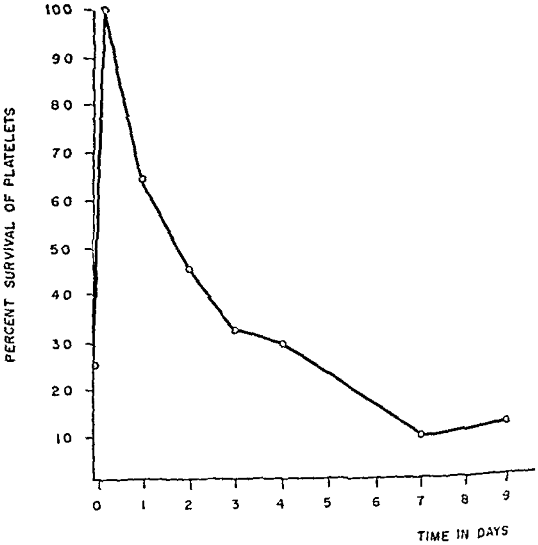

Fig. 7.

Fig. 6.—The survival of transfused radioactive platelets in recipient Dog 3.

Fig. 7.—The survival of transfused radioactive platelets in a normal human recipient (Patient 1).

The survival of radioactive platelets in a third dog is shown in Fig. 6. Here again there is evidence of sequestration, followed by an apparently exponential curve of decay. The half-life was short, 1½ days, and the 10 per cent level was reached on day 6.

Fig. 7 shows the typical survival of radioactive platelets in a normal *human* recipient. The amount of platelet sequestration that occurred after the transfusion is striking. The curve of decay in the human subject also appears to be exponential. The half-life was 2 days, and the 10 per cent level was reached on day 7.

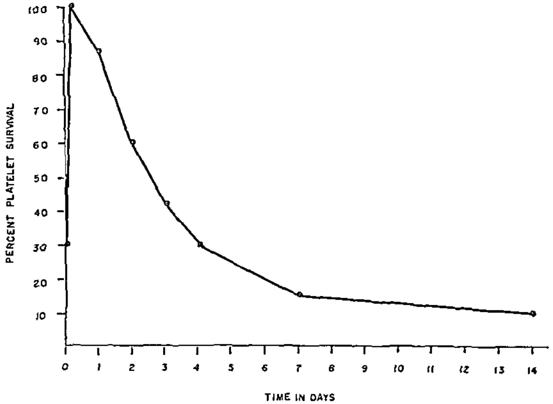

FIG. 8—The survival of transfused radioactive platelets in a normal human recipient (Patient 2).

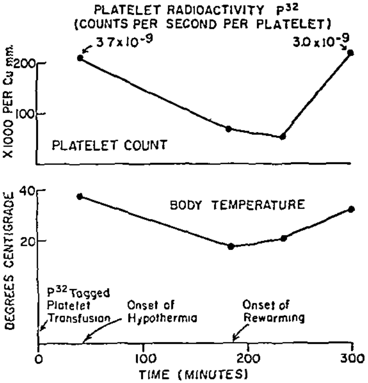

FIG. 9—The sequestration of platelets during artificial hypothermia. Radioactive platelets were transfused into the recipient dog before his temperature was lowered.

Fig. 8 shows a second example of platelet survival in a normal human. Again there is an exponential curve. The half-life was 2½ days, and the 10 per cent level is reached on day 14. The sequestration was even more marked than in the dogs. We belive this to be the first time that platelet sequestration has been demonstrated.

Using the technique of tagging platelets with P^{32}, we have demonstrated a second example of platelet sequestration. In dogs undergoing deep hypothermia, we found nearly complete disappearance of platelets as the animals were cooled to 20° C., and a reappearance of platelets when they were rewarmed.[12] We transfused radioactive platelets into a dog which was then exposed to hypothermia (Fig. 9). The animal's platelet count dropped nearly to zero. On rewarming, the platelet count returned to normal. The radioactivity in samples of platelets obtained before and after chilling was nearly the same. The platelets which appeared after hypothermic thrombocytopenia were the same platelets which were in the blood before the dog was chilled. During deep hypothermia, platelets are sequestered.

SUMMARY

A new method of tagging platelets in vivo with P^{32} is presented. It is accomplished by injecting P^{32} into a donor, waiting until his platelet population contains a significant concentration of intrinsically labeled platelets and then transfusing his whole blood into a compatible recipient. The method has the advantage of requiring only minimal manipulation of the platelets. The curve of decay representing platelet destruction appears to be exponential both in humans and in dogs. The half-life is 1½ to 3 days, and the 10 per cent level of survival is reached in 6 to 14 days. Both in humans and in dogs there is evidence of a transient sequestration of platelets in the early posttransfusion period. A second example of platelet sequestration has been demonstrated by applying this technique of platelet tagging to the study of thrombocytopenia in hypothermic dogs.

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THE PERSISTENCE IN THE BLOOD OF THE RADIOACTIVE LABEL OF ALBUMINS, GAMMA GLOBULINS, AND GLOBULINS OF INTERMEDIATE MOBILITY

VI. The Uptake Plots of S^{35} Into Serum Protein Fractions During the First 24 Hours After Oral Administration of Labeled Methionine and Cysteine

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S. Howard Armstrong, Jr., M.D.
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WHEN our studies[1, 2] of die-away plots (representing the metabolic degradation of S^{35} autosynthesized protein fractions) did not permit sure metabolic or clinical interpretation, our attention was directed to uptake plots (representing the incorporation of S^{35} into plasma protein fractions during the first 24 hours after oral administration) as a possible means of estimating synthesis rates. While free from the unmeasurable complication of isotope reutilization encountered in die-away plots, and while less tedious in execution, uptake plots nevertheless have all the pitfalls of tolerance tests.

It is the purpose of this paper: (1) to report our preliminary findings on the uptake of S^{35} into certain serum protein fractions during the first 24 hours after oral administration of labeled methionine and cysteine, and (2) to evaluate these findings in the light of clinical variables other than synthesis rates which may determine the form of an uptake plot, in order to see whether or not such variables (which are often hard to control) are sufficiently important to warrant promise for such plots in the clinical investigation of plasma protein metabolism.

METHODS

Subjects.—The 12 subjects studied fall into 4 diagnostic categories (Table I). These categories were chosen to cut across the possible clinical correlations for an uptake test.

The subjects with proved pancreatic disease represent deficiencies in dietary protein hydrolysis of varying severity as indicated by a range of serum albumin levels from 0.7 Gm./100 c.c. (Patient T. N.) to 3.4 Gm./100 c.c. (Au. J.). The ability to absorb the I^{131} label from oral albumin was, therefore, also measured on each patient in this group, as specified below and reported in Table I.

The patients in the second diagnostic category of Table I represent small bowel malabsorption of etiology ranging from the known (postoperative short bowel syndrome with

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stricture) to the unknown (regional ileitis and idiopathic nontropical sprue). Here also a range in serum albumin levels (0.8 Gm./100 c.c., Patient W. S. to 2.7 Gm./100 c.c., Patient J. S.) indicates varying severity of this defect.

All patients in the first two diagnostic categories exhibited steatorrhea.

The third category, which bears in Table I the heading "hypo- or dysproteinemias," includes the following cases.

1. Patient T. C. was deficient chiefly in the albumin fraction. This invited comparison with the findings of Kinsell and co-workers[3] in their patient with diagnosis idiopathic "hypoproteinemia." A previously published[4] die-away plot following I.V. albumin I^{131} for this patient yielded a shortened apparent turnover time for albumins comparable to the figure recently published for this diagnosis by Schwartz and Thomsen in Denmark,[5] as well as a depressed total exchangeable albumin pool.*

2. Patient A. R. exhibited deficiency chiefly in the gamma globulin fraction.† His idiopathically low level did not, however, approach the complete agammaglobulinemia of the patient we have previously reported in whom we could demonstrate no S^{35} uptake whatsoever in the gamma position.[6]

TABLE I*

DIAGNOSTIC GROUPS	PATIENT	MAXIMUM ALBUMIN I^{131} UPTAKE (% DOSE)	MAXIMUM S^{35} UPTAKE†			
			TOTAL PROTEIN	ALBUMINS	INTERMEDIATE GLOBULINS	GAMMA GLOBULINS
Pancreatic disease	T. N.	1.5	9,600	2,300	5,800	1,400
	Al. J	4.0‡	3,200	1.310	1,640	720
	Au. J.	4.0	3,500	1,350	2,000	500
	M. L.	6.5	3,400	1,200	1,800	550
	V. H.	4.5	3,600	1,280	1,750	480
Small intestinal malabsorption	L. D.	18.0	5,500	1,400	2,600	1,120
	W. S. (1)§	--	5,600	900	2,600	980
	(2)	9.0	5,100	1,800	3,400	600
	J. S.	12.0	3,500	1,050	2,750	400
Hypo- or dysproteinemias	T. C. (albumins)‖	11.2	9,200	6,500	3,300	1,100
	A. R. (γ globulins)	--	3,260	1,048	1,928	584
	J. F. (γ globulins) ¶	--	1,968	1,156	1,090	270
Nutritionally normal	J. M.§	15.1	5,950	2,600	2,200	750

*The diagnoses and paper electrophoretic albumin and globulin levels for 8 of the patients in Table I are given in Table I of the preceding paper in this series.[1] (Also, in Fig. 1 of this reference, die-away plots are presented for albumins and gamma globulins of 5 of these patients.)

In addition, Patient V. H. presented chronic calcific pancreatitis (autopsy). His serum protein levels were: total protein 6.0, albumin 2.8, and globulin 3.2 Gm./100 c.c. Patient J. S. presented regional ileitis (surgery). His serum protein levels were: total protein 6.0, albumin 2.7, and globulin, 3.3 Gm./100 c.c.

†This uptake (corrected counts per minute) is corrected to a standard 0.5 mc. oral dosage and is proportional to the true specific radioactivity in terms of mc./Gm.

‡Postpancreatectomy study. Preoperative albumin I^{131} uptake was 15.0 per cent.

§At initial labeling,[1] electrophoretic partition showed total protein 3.0, albumin 0.8, and gamma globulins 1.2 Gm./100 c.c. During remission, repeat labeling,[2] showed total protein 5.5, albumin 2.2, and gamma globulin 1.2 Gm./100 c.c.

‖Labeled with S^{35} cysteine 0.5 mc. All others received S^{35} methionine 0.5 mc.

¶This patient with multiple myeloma had, in addition, an "M" protein of 5.3 Gm./100 c.c., with a maximum S^{35} uptake of 342 c.p.m.

3. In Patient J. F. the deficiency was also chiefly in the gamma fraction, but was accompanied by a large fraction of slightly faster mobility characteristic of multiple myeloma.

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*Reference 4 discusses the role of noncooperation with diabetic management in the etiology of this [illegible] However, continuous ward observation for a period of nearly two [illegible] ts at supplementation, has led us to the belief that there is a further [illegible]

†Judged by absence of isohemagglutinins and by history of multiple bacterial infections, this patient approached the state of "immunologic paralysis" although he demonstrated cutaneous sensitivity to tuberculin.

Finally there is included one metabolically normal subject (J M) whose die-away plots followed both the oral S^{35} and the intravenous I^{131} fractions we have already published 1.

Administration of Labels and Supplements, Radioactivity Measurements—Two subjects (T C and J M) received oral S^{35} cysteine, 0 50 mc All others received oral S^{35} methionine, 0 50 mc Supplemental protein hydrolysates were given orally, in 2 5 Gm doses at the time of labeling Prolonged supplementation was not given Sera were drawn at 1, 2, 4, 8, and 24 hour intervals Methods of fractionation, extraction, measurement of component concentrations and radioactivity have been described previously No significant differences were observed between radioactivity measurements on specimens before and after dialysis 6

Radioactivity uptakes were plotted on regular graph paper as corrected counts per minute against time in hours By the corrections used these values are proportional to the specific radioactivities of the fractions in question (in terms of counts per minute per gram protein) and are corrected to a standard oral radioactivity dosage of 0 50 mc

A maximum of 26,000 counts per minute in the "total protein" column of Table I would be achieved if 100 per cent of the oral dosage were present in a plasma volume of 2500 c c at the time of the uptake plot maximum Individual plasma volume measurements were not carried out in this study

The albumin I^{131} absorbtion test was performed as described by Freeark, Kozoll, and Meyer 8 Normal maximal absorption with this procedure is 14 per cent (range, 10 to 22 per cent) of the given dose

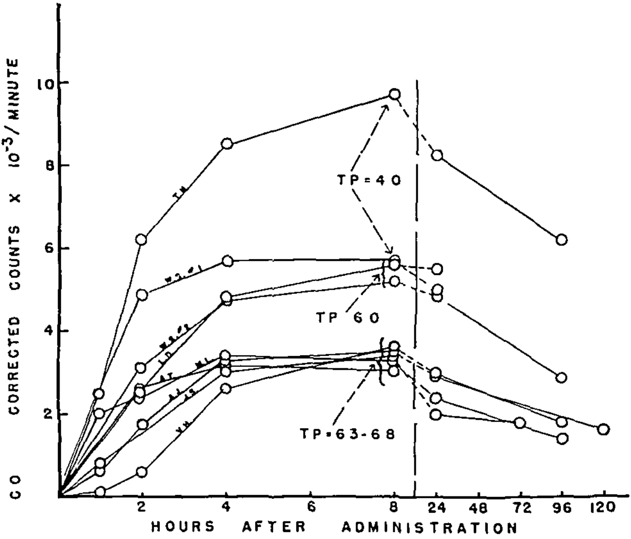

Fig 1—The incorporation of S^{35} methionine (500 μc) into unfractionated serum proteins during the first 24 hours after oral administration The initials of specific patients are indicated on individual plots between hours 2 and 4 the total protein ranges between hours 6 and 8 above the abscissa

RESULTS

Uptake of S^{35} by Unfractionated Serum Proteins.—The incorporation of S^{35} into the total serum protein reaches a maximum eight hours after oral administration. Fig. 1 illustrates this by the complete plots for 9 of the 13 studies whose maxima are given in Table I. Not infrequently a maximum level is reached at 4 hours. Kinsell[3] obtained similar results with intravenous S^{35} methionine.

Inspection of the left side of Fig. 1 indicates a lack of correlation between the initial steepness of plot and the type of digestive disturbance. The plots of greatest (T. N.) and least (V. H.) steepness both correspond to a common pathogenesis, pancreatic disease. The next steepest (W. S., 1) and the next flattest (J. S.) correspond to syndromes in which local absorptive problems might be expected to influence a tolerance test.

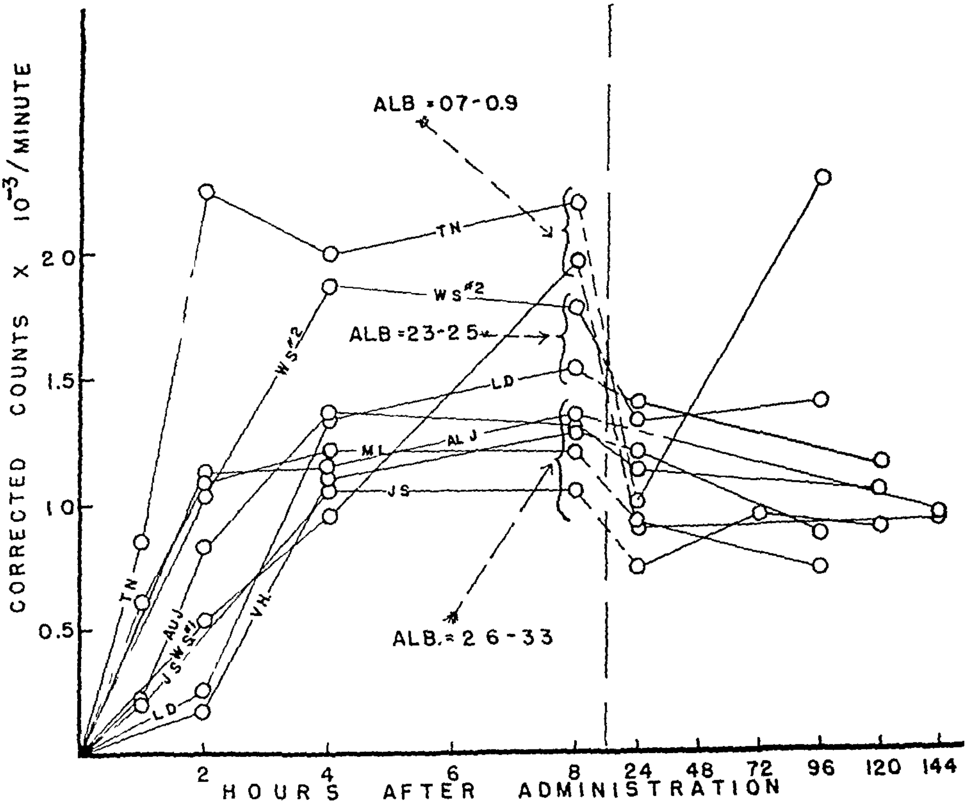

Fig. 2.—The incorporation of S^{35} methionine (500 μc) into the serum albumins during the first 24 hours after oral administration. Patients and serum albumin concentrations ranges are indicated essentially as in Fig. 1.

Inspection of Fig. 1 and Table I, shows that the maxima of the uptake plots may be inversely correlated with the level of serum total proteins. The highest maximum occurred in T. N., whose total protein was 4.0 Gm./100 c.c. In patient T. C. (not shown in Fig. 1), with a total protein of 3.7 Gm./100 c.c., a comparably high maximum is seen. L. D. shows a similar though somewhat lower maximum. Kinsell's demonstration[3] of an elevated uptake of S^{35} into the total plasma protein of a patient with idiopathic hypoproteinemia is thus extended to hypoproteinemia of another etiology.

However, this correlation does not always hold even in this small series. Patient W. S. was studied twice. In the first study, his total protein was 3.0 Gm./100 c.c., while in the second study, during a period of remission, his total protein had risen to 5.5 Gm. per cent after intensive nutritional therapy on the ward. The 8 hour uptake maximum, however, was alike in both studies.

Uptake of S^{35} into Serum Albumins.—The highest maxima occurred in uptake plots of patients with the greatest depressions of the serum albumin level (e.g., Patient T. N. in Fig. 2 with an albumin level of 0.7 Gm./100 c.c.) In Patient T. C. (not illustrated), with an albumin level of 1.3 Gm./100 c.c., an uptake three times as great as this was achieved after oral S^{35} cysteine. Again, Patient W. S., in duplicate studies, presented similar uptakes, although his albumin level had risen from 0.9 to 2.3 Gm./100 c.c. For patients with lesser degree of hypoalbuminemia, the corresponding differences in degree of uptake were less significant. As with the total serum proteins, there thus appears a rough inverse proportionality of uptake maximum to the level of serum albumin. (Fig. 2 presents complete plots for 9 of the studies whose maxima are given in Table I.)

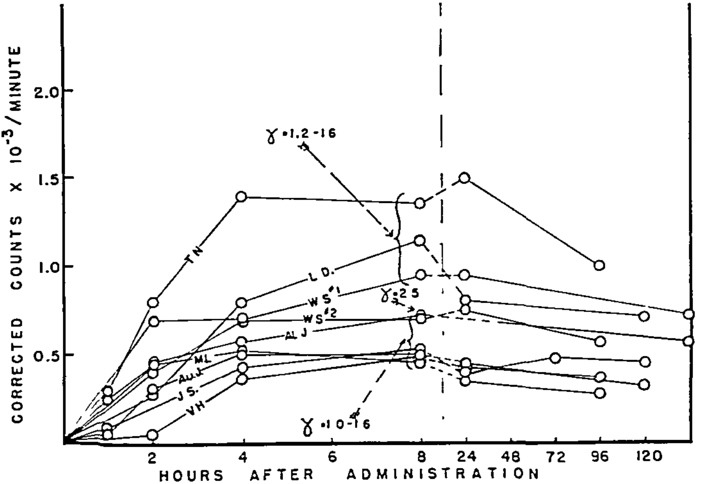

FIG. 3.—The incorporation of S^{35} methionine (500 μc) into the gamma globulins during the first 24 hours after oral administration Note, in contrast to Figs. 1 and 2, that the order of maxima of the plots shows no correlation with order of gamma globulin concentrations.

Uptake of S^{35} into Gamma Globulins.—While in general uptake maxima were significantly lower than for corresponding albumins (compare Figs. 2 and 3), in contrast to the albumins, no relationship could be observed between these maxima and the levels of serum gamma globulin (Fig. 3 and Table I). The highest maximum occurred in nutritionally normal Patient J. M. (Fig. 4, note S^{35}-cysteine label). However, both T. C. and T. N. (who showed highest total protein and albumin maxima) also presented high gamma globulin maxima when compared to the others in the group.

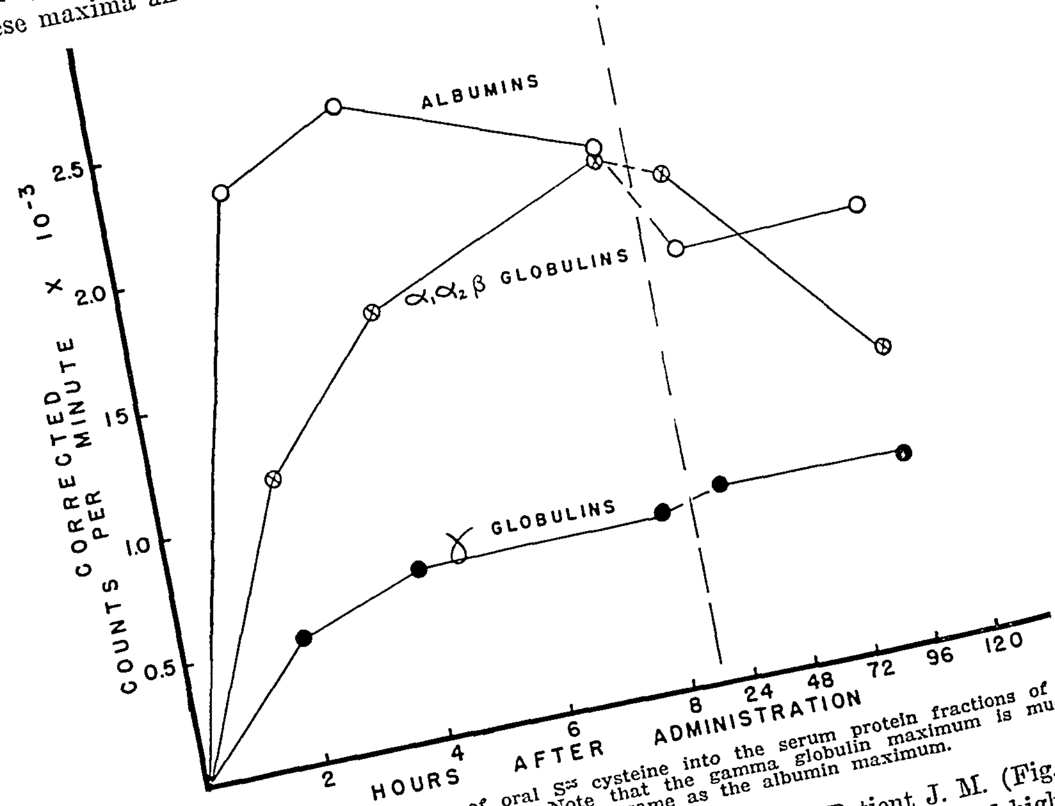

Fig. 4.—The incorporation of oral S^{35} cysteine into the serum protein fractions of a nutritionally normal patient (J. M.). Note that the gamma globulin maximum is much lower, and the alpha-beta maximum about the same as the albumin maximum.

Uptake of S^{35} into Globulins of Intermediate Mobility.—The plot maxima for this crude mixture of alpha-1, alpha-2, and beta globulins (Table I) have been in general greater than the corresponding albumins maxima save in the two instances where S^{35} cysteine was used as the oral label. Fig. 4 presents for Patient J. M., uptake plots which illustrate this point in comparison with those in Fig. 5 (Patient J. S.).

While these high maxima are of interest in the light of the short apparent turnover times we have already published for this fraction, because of its crudity we will limit our discussion at this time to the albumins and gamma globulins.

I^{131} Uptake from Oral Albumin I^{131}.—The results of this procedure are given in Table I. All the patients with chronic pancreatic disease showed difficulty in absorbing an albumin I^{131} test meal. Patient L. D., with idiopathic sprue, is of especial interest since she not only did not show any difficulty in absorbing the label from oral albumin I^{131}, but also little difficulty in S^{35} amino acid uptake. The same is true for Patient J. S. (regional ileitis).

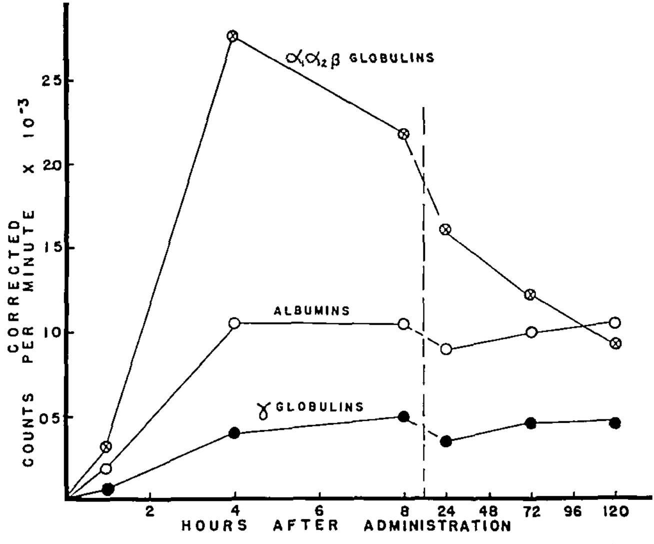

FIG. 5.—Incorporation of oral S^{35} methionine into the serum protein fractions of a patient (J. S.) with regional ileitis and steatorrhea. Note that while gamma and alpha beta maxima are similar to Fig. 4 the albumin maximum represents less than one half the Fig. 4 value.

DISCUSSION

Given a standard dosage, the form of an oral tolerance plot is clearly a function of rates of absorption as influenced by both gastrointestinal motility and local conditions. Our oral uptake plots for total proteins resemble those published by Kinsell and co-workers following intravenous administration of S^{35} methionine. The resemblance suggests such rapid intestinal absorption that oral labeled amino acids probably do not hold as promising a future role in clinical elucidation of absorption defects as do the I^{131} labeled lipids.[9-11]

Further groups of variables which influence the form of the plot include (a) rates of equilibration with extravascular body compartment, (b) rates of excretion by the kidney, and (c) rates of metabolic turnover. Such a range of uncontrolled variation makes it amazing that tolerance tests have been capable of any useful clinical correlation. When an isotopic label is used a further variable is emphasized: namely, the circulating pool size of the unlabeled component to which the newly introduced label is added.

Thus given hypothetically identical dosages, absorptions, and rates of incorporation into serum albumin, if the liver delivers the same amount of S^{35} albumin into identical plasma volumes of 2 subjects, one with an albumin level of 3 Gm./100 ml., the other with a level of 1 Gm./100 ml., the specific radioactivities, and thus probably the plot maxima, will be one third as high for the first subject as for the second. This problem in more complex form is clearly recognized by Gould and co-workers[12] as a limitation in clinical estimation of the rate of cholesterol synthesis from uptake plot maxima following C^{14} administration. These authors proposed correction procedures which might give their tolerance test "value as a clinical laboratory test." Kinsell used such a correction in interpreting the twice normal S^{35} uptake maxima in his patient with idiopathic hypoproteinemia and concluded that the defect is "hypercatabolism not compensated by hyperanabolism." If we apply correction factors based on individual serum electrophoretic levels to the maxima of Table I, the resultant maxima should be, to a first approximation, independent of pool size, and thus a rough measure of the synthesis rate of the component in question.

TABLE II

PATIENT	LEVELS (GM./100 C.C.)			CORRECTED RADIOACTIVITY MAXIMA			SUGGESTED INTERPRETATION
	ALBUMINS	GLOBULINS	OTHER	ALBUMINS	GLOBULINS	OTHER	
				Hypo-albuminemias			
T. N.	0.7	1.6	--	420	1,720	--	Depressed albumins synthesis and accelerated gamma globulin synthesis related to pancreatic disease and hepatic disease
W. S.(1)	0.8	0.9	--	190	900	--	Depressed albumin synthesis related to malnutrition of chronic small bowel disease, restored toward normal by dietary therapy
W. S.(2)	2.2	0.9	--	1,030	550	--	
T. C.	1.3	0.7	--	2,200	810	--	Accelerated albumin synthesis in the face of chronic hypoproteinemia resistant to dietary therapy, pointing to a hypercatabolic albumin utilization without comparable gamma globulin abnormality
				Hypogammaglobulinemias			
A. R.	4.2	0.3*	--	1,140	140	--	Depressed gamma globulins synthesis in the face of normal albumin synthesis
J. F.	2.1	0.55	5.3†	632	115	1,395†	Depressed gamma globulins synthesis in the face of an elevated synthesis of the abnormal "M" protein; probable depression of albumin synthesis

*This value was obtained at time of uptake study. Subsequent serial observations have yielded a range between 0.1 and 0.4 Gm./100 c.c.

†"M" protein, intermediate in mobility between gamma and beta globulins. The correction factor used was based on the normal circulating gamma globulins pool. If the circulating albumins pool is used for the correction factor, the correct maximum becomes 470 counts per minute.

The roughness of the first approximation is indicated by the fact that the correction factors neglect unmeasured variability in plasma volume and in renal and fecal losses of the labeled amino acid as well as losses of the labeled protein component to extravascular compartments.*

Table II (left side) presents the results of such correction to the albumin and gamma uptake maxima for the 5 patients of Table I for which correction factors are greatest.† These 5 patients include 3 hypoalbuminemias and 2 hypogammaglobulinemias.

It will be seen, for the former, that a deficient level has been found associated with uptake maxima both exceedingly low and quite high. Suggested interpretations (Table II, right side) indicate our belief in the possibility of thus differentiating a hypercatabolic from a deficient level due to other causes.

Maxima for the hypogammaglobulinemias have been exceedingly low. It is tempting to interpret the low gamma globulin uptake in patient J. F. (myeloma) as an incapacity of the plasma cell mass to put out normal gamma globulins in the face of synthesis of "M" protein. In favor of this is the high "M" protein uptake and the normal albumin uptake (Table II, bottom line).

We recognize that the maxima of these uptake plots may not reflect capacity of a given organ system for synthesis of a given protein component. For avidity in another organ system, rather that incapacity in the one, may so influence the distribution of labeled amino acid as to make estimates of defects in synthesis capacity undetectable.

SUMMARY

Because of our failure to achieve reliable metabolic and clinical interpretations of the die-away plots of autosynthesized plasma protein fractions following oral S^{35} amino acid administration, our attention has turned to the uptake plots of radioactivity during the first 24 hours after feeding the label. Such plots are subject to the multiple variables and interpretive pitfalls of tolerance tests, and are discussed in these terms.

Plots are reported for a small pilot group of twelve subjects divided between four diagnostic categories. The subjects were chosen (a) to represent samples of the clinical spectra of hypoalbuminemia and hypogammaglobulinemia, (b) to include a range of hypoalbuminemias on the basis of chronic pancreatic deficiency, as well as the "idiopathic" and small intestinal malabsorption type, and (c) to include subjects with normal serum concentrations of gamma globulins in the presence of albumin deficiency, and vice versa.

No significant dialyzable radioactivity appeared in the blood stream after the oral S^{35} amino acids. This suggests very rapid incorporation of absorbed dosage into serum proteins and/or segregation intracellularly. Nondialyzable serum radioactivity appeared within an hour.

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*The latter losses are probably sufficiently small as compared to the differences in uptake maxima as to warrant neglect of the complex mathematical treatment[13] at this stage of our work.

†The correction factors by which the Table I maxima are multiplied represent the ratios of the observed electrophoretic concentration to the normal serum concentration of that component.

Based on standardized label dosage, specific radioactivity maxima were achieved in about eight hours and were, in all instances, higher for albumins than for corresponding serum gamma globulins.

While it proved possible to distinguish pancreatic hypoalbuminemias from both small intestinal malabsorption and from a normal subject on the basis of absorbed radioactivity from the oral intact protein albumin I^{131}, no such differentiation could be accomplished by the initial slopes (representing rapidity of absorption) of the S^{35} amino acid uptake plots.

When uncorrected for the circulating pool of unlabeled protein component to which the newly synthesized S^{35} protein is added, albumins uptake maxima, in general, appeared inversely proportional to the degree of hypoalbuminemia.

While reflected in the uptake maxima for unfractionated serum proteins, such an inverse relationship did not appear applicable for gamma globulins.

When *maxima for the albumins and gamma globulins were subjected to* an arbitrarily estimated correction for unlabeled circulating pools (a) the maxima for hypogammaglobulinemias were strikingly depressed as compared to the maxima for those subjects with a normal serum concentration of this electrophoretic component, while; (b) the instances of hypoalbuminemia included maxima both strikingly lower and considerably higher than those for subjects with more normal serum albumin concentrations.

It is suggested that these maxima may bear a rough relation to the rates of synthesis of the component in question, and that the spread of albumins maxima suggests the possibility of clinically distinguishing "hypercatabolism" from other mechanisms.

While it is recognized that uptake plot maxima can never be a measure of maximum synthesizing *capacity* of an organ system, and merely represent a very rough approximation to the resultant of many simultaneous processes, nevertheless, these findings seem sufficiently encouraging to warrant further clinical exploration in a larger series of defects in the metabolism of plasma proteins.

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THE EFFECT OF L-NOREPINEPHRINE ON URINE COMPOSITION AND ACID-BASE EQUILIBRIUM IN MAN

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THE adrenal medullary hormones, l-epinephrine and l-norepinephrine, elicit acute alterations in renal function when administered in physiologic dosage to normal man. Both induce intrarenal vasoconstriction and a reduction in renal blood flow, a sustained or increased urine volume, and an augmented tubular reabsorption of sodium and potassium.[1-3] The effect on urine flow has been attributed, at least in part, to suppression of the secretion of the antidiuretic hormone of the posterior pituitary.[4, 5] The factors contributing to the accelerated tubular electrolyte transport are not clear. This action of the medullary hormones appears to be independent of their pressor activity and also of secondary adrenal cortical secretion and renal neurogenic activity.[4] The electrolyte retaining effect of these substances has not, however, been demonstrated independently of the accompanying intrarenal circulatory adjustments[4] nor has the change in tubular activity been separated from the systemic metabolic alterations elicited by these hormones.

These alterations in sodium and potassium output have been reported to occur in association with other changes in urine composition. Chloride excretion has been found to diminish[3] and urinary pH to increase.[6] However, a systematic study of the effects of the medullary hormones on urinary acid-base excretion has not been made. Alterations in plasma acid-base balance, consisting of an early, transient respiratory alkalosis[7, 8] and a later and more prolonged metabolic acidosis,[8-10] are elicited by these agents, but the relationship between these adjustments and the changes in urine composition is not known.

These considerations lead to the present investigation which was undertaken in order to determine the effects of l-norepinephrine on urinary acid-base excretion and to study the relationship between the changes in urine and plasma composition.

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METHOD

The effects of l-norepinephrine on urinary and plasma acid-base composition were determined in 12 normal human subjects. The effects of ephedrine sulfate were observed in three additional subjects. The subjects varied in age from 21 to 58 years and were free from apparent cardiovascular or renal disease. Each was maintained on a regular hospital diet containing approximately 10 Gm. sodium chloride daily.

All studies were made in the morning after an overnight fast and after the subject had ingested 500 ml. of water. Urine was collected under mineral oil through a multi-eyed urethral catheter. At the mid-point of each period enough urine was collected for the determination of pH and CO_2 content. At the end of each period the bladder was irrigated with distilled water and emptied as completely as possible by manual compression. Blood was withdrawn anaerobically at appropriate intervals from a brachial artery (10 subjects) into oiled heparinized syringes. In 5 subjects (D. H., E. P., E. C., J. S., and M. G.) samples of blood were taken from an antecubital vein following immersion of the forearm in water at 47° C. for 10 minutes. Blood was centrifuged immediately and the plasma was stored in a refrigerator until analysis could be made, which was usually within 4 hours.

Glomerular filtration rate was measured as the inulin clearance.[11] The pH of plasma and urine were determined anaerobically at room temperature with a Cambridge glass electrode pH meter. Plasma pH was corrected to body temperature using a factor of 0.01 pH unit per degree centigrade.[12] Urine pH was not corrected to body temperature.[12] Urine and plasma were analyzed for total CO_2 content by the method of Van Slyke and Neill.[13] Bicarbonate and carbonic acid concentrations were calculated from the Henderson-Hasselbach equation using a value of 6.1 for pKa'. Carbon dioxide tension was calculated from the carbonic acid content, using a value of 0.301 and 0.0309 for α in plasma and urine, respectively.[14] Urinary titratable acid was determined with an external glass electrode, titrating to the pH of plasma with 0.01 N NaOH. The ammonia content of urine was determined by a modification of the method of Van Slyke and Cullen.[15] The urine of two subjects (S. G. and E. T.) was analyzed for organic acids by the method of Van Slyke and Palmer.[16] However, a correction for creatinine was not made. Urine and plasma were analyzed for chloride by the method of Schales and Schales[17], for sodium and potassium in an internal standard flame photometer, and for inorganic phosphate by the method of Fiske and Subbarow.[18]

Following the determination of control values during 2 or 3 successive 10- to 20-minute intervals, l-norepinephrine (8 μg per ml. 5 per cent dextrose in water) or ephedrine sulfate (2 μg per ml.) was administered intravenously at a rate sufficient to maintain systolic blood pressure, determined sphygmomanometrically, at least 25 to 50 mm. Hg above control values. The amount of drug employed varied from 1 to 30 μg per minute of l-norepinephrine and from 0.5 to 6.0 mg. per minute of ephedrine. Blood pressure was allowed to stabilize from 10 to 20 minutes and urine collected during this interval was discarded. Two or three clearance periods, 10 to 20 minutes in length, were then obtained during infusion of the pressor agent. One or two "recovery" periods were obtained after the drug was discontinued in all subjects but D. C. and D. T. Blood pressure and pulse rate were recorded every one to two minutes during and following infusion of the pressor agent.

The response to l-norepinephrine was examined in 6 subjects who were in normal acid-base balance and in 3 who had been given 6 to 12 Gm. of ammonium chloride orally over an 8- to 12-hour period on the day prior to the study. Ammonium chloride was not given within a minimum of 4 hours, and usually not within 8 hours of the beginning of the experiment.

Three additional subjects received an infusion of 2.25 per cent sodium bicarbonate at a rate (4 ml. per minute) sufficient to increase progressively the CO_2 content of plasma throughout the study. Two of these subjects (F. G. and E. P.) had received ammonium chloride the day previously. The response to ephedrine was studied in three subjects, two of whom (E. C. and J. S.) had received ammonium chloride.

RESULTS

Response to l-Norepinephrine.—The changes in acid-base composition of urine and plasma induced by l-norepinephrine are presented in Table I. Plasma

TABLE I. THE EFFECT OF L NOREPINEPHRINE ON

SUBJECT	SEX	AGE	S.A.	PROCEDURE	B.P. (mM./Hg)	P.R.	PLASMA Na (mEq./L.)	K (mEq./L.)	Cl (mEq./L.)	PO_4 (mM./L.)	HCO_3 (mM./L.)	pCO_2 (mM./Hg)	pH (units)
B.W.	M	36	1.96	Control	120/70	52	135	3.8	108	0.89	25.6	39.5	7.44
				Infusion	159/81	50	135	3.6	106	0.72	24.2	37.5	7.44
				Recovery*	124/70	70	137	--	108	0.69	24.2	36.2	7.45
D.H.	F	26	1.74	Control	98/58	80	136	3.7	109	0.77	24.8	43.2	7.38
				Infusion	144/88	54	129	3.8	106	0.72	23.8	41.5	7.39
				Recovery	96/50	66	129	3.5	105	0.75	24.3	41.9	7.38
M.G.	F	21	1.60	Control	110/60	76	134	3.6	110	--	25.5	35.9	7.49
				Infusion	146/88	60	136	3.5	106	--	23.8	33.2	7.49
				Recovery	106/68	72	135	3.5	110	--	25.4	34.2	7.48
S.G.	M	40	1.62	Control	109/70	77	137	4.8	109	1.00	22.8	35.6	7.43
				Infusion	170/100	60	134	4.7	107	0.80	21.5	32.2	7.44
				Recovery	110/68	71	134	4.6	103	0.80	22.4	33.6	7.44
O.G.	M	31	1.68	Control	98/68	72	142	4.1	108	1.16	26.3	39.9	7.44
				Infusion	136/80	64	141	3.9	106	1.09	25.6	39.2	7.43
				Recovery*	90/60	72	139	4.1	106	1.07	26.1	39.5	7.43
D.C.	M	49	1.68	Control	100/70	80	131	4.2	104	1.07	25.2	41.9	7.44
				Infusion	141/92	72	133	4.2	106	1.11	24.2	39.9	7.41
A.C.	M	37	1.88	Control	107/68	54	132	4.2	105	0.90	25.6	45.2	7.37
				Infusion	168/90	48	132	4.2	107	1.00	25.1	44.2	7.37
				Recovery	112/70	54	134	4.0	105	1.00	25.0	43.5	7.37
E.T.	M	37	1.79	Control	120/72	90	133	4.3	105	0.83	21.5	38.2	7.35
				Infusion	172/90	66	133	4.2	105	0.87	19.3	41.5	7.28
				Recovery	120/70	82	133	4.1	109	0.85	21.0	38.2	7.36
A.M.	F	42	1.66	Control	120/78	96	131	3.9	112	0.87	17.9	34.2	7.34
				Infusion	164/90	78	129	4.1	110	0.93	17.1	32.2	7.32
				Recovery	106/76	118	131	4.1	112	0.83	17.9	34.6	7.32

Key to abbreviations: S.A. = body surface area (M^2); P.R. = pulse rate; V = urine flow (ml./min.); GFR = glomerular filtration rate, inulin clearance (ml./min.); T.A. = titratable acid (mEq./min.). A. C., E. T., and A. M. had received ammonium chloride on the day prior to the study.

*All values are averages of two or more determinations except in an
asterisk L-norepinephrine was administered during the "infusion" pe to
produce the desired elevation of arterial pressure, which was measured The
values of various urinary constituents represent excretory rates except for pH and pCO_2.

bicarbonate content decreased slightly, carbon dioxide tension fell, and the acidity of plasma was unchanged. The reduction in bicarbonate concentration varied from 0.7 to 1.7 meq. per liter, averaged 1.1 meq. per liter, and was highly significant ($p < 0.01$). During recovery all values returned promptly toward control levels. The plasma concentrations of sodium, potassium, chloride, and phosphate did not change.

The urinary excretion of sodium and potassium diminished during l-norepinephrine infusion in 4 of 6 subjects. This reduction in cation excretion was accompanied largely by a diminished excretion of chloride, and in D. H. and M. G. by a reduction in bicarbonate output as well. Titratable acid, inorganic phosphate, and ammonium excretion and urinary pH showed no consistent change. In some instances (D. H., S. G., A. C., and D. C.) bicarbonate output appeared to vary with changes in urine flow rather than with changes in sodium and potassium output. This correlation has been noted by others.[12, 19] Organic acid excretion, not presented in the table, did not change in the two subjects (S. G. and

Acid Base Equilibrium and Urine Composition*

	URINE									
GFR (ML/MIN)	V (ML/MIN)	pH (units)	Na (μEq/MIN)	K (μEq/MIN)	Cl (μEq/MIN)	PO_4 (μM/MIN)	HCO_3 (μM/MIN)	pCO_2 (mM/Hg)	T A (μEq/MIN)	NH_4 (μEq/MIN)
114	10 4	5 61	101 5	43 3	0	8 3	5 6	56 0	25 3	38 9
114	10 2	5 08	86 8	37 4	0	6 1	1 7	55 5	33 9	41 3
101	7 7	5 29	95 8	24 3	0	6 0	2 0	51 5	22 5	32 1
123	9 7	6 72	137 7	72 7	77 9	6 9	45 1	35 8	5 0	14 8
95	5 3	6 60	90 3	30 2	64 0	6 1	18 5	35 8	6 5	24 3
112	3 0	6 23	52 9	17 7	41 9	7 8	6 4	35 2	8 4	18 3
153	6 5	6 59	271 8	70 3	200 0	--	21 9	34 2	8 9	32 9
116	6 6	5 82	206 0	55 4	103 9	--	4 1	38 2	14 9	34 7
127	7 4	5 68	245 8	60 0	141 5	--	3 4	36 9	19 0	36 8
104	4 7	6 75	75 4	49 4	71 7	3 7	18 7	30 8	--	13 5
150	7 0	6 82	84 7	54 6	88 8	1 6	32 0	28 4	--	10 3
108	4 2	6 17	28 7	38 3	40 3	1 2	6 1	49 9	--	11 2
77	5 9	6 35	68 3	65 9	108 0	7 8	11 1	34 3	6 3	19 9
107	7 1	6 18	79 9	53 3	118 0	10 0	9 0	34 7	8 6	22 1
71	3 1	6 08	82 1	53 7	128 0	13 2	3 0	44 7	8 3	10 1
124	3 1	6 68	145 0	38 8	126 0	8 6	23 4	63 4	6 7	13 9
139	7 0	6 48	134 0	37 7	110 0	14 3	26 6	54 9	17 7	17 4
136	4 4	5 35	205 2	79 2	301 7	5 7	1 2	43 9	17 5	45 0
133	9 2	5 55	144 6	68 7	207 7	11 4	4 7	56 7	22 5	44 0
131	6 8	5 61	148 9	55 0	204 8	12 5	1 8	44 3	14 0	46 0
140	7 2	5 79	262 8	69 3	353 3	0	5 9	49 0	--	45 7
120	7 9	5 82	76 0	40 9	136 5	0	6 1	47 5	--	45 3
178	3 4	5 41	137 2	44 0	207 9	1 6	2 0	36 4	--	20 3
140	11 0	5 29	193 0	41 2	250 0	14 3	2 3	43 4	22 9	32 8
61	10 2	5 13	124 8	36 9	165 9	17 1	1 5	42 3	26 2	31 5
118	3 9	5 17	91 3	20 2	130 9	10 9	0 5	35 5	16 6	25 1

E T) in whom it was measured Gross deficits of either cation or anion output were not encountered The anion deficit apparent in subject B W was the result of the presence of Diodrast which was administered as part of another study Subject M G also had received Diodrast

In two subjects (S G and O G) urinary electrolyte excretion, which was low in the control periods, increased in association with a rise in glomerular filtration rate This response was unlike that usually elicited by the adrenal medullary hormones However, in these subjects electrolyte output increased less than filtration, suggesting an augmented tubular reabsorption per unit of filtrate No consistent alteration in urinary acid base equilibrium was noted in these subjects

The withdrawal of l norepinephrine was usually followed by a reduction in urine volume (Tables I and II), which, in most, but not all, instances had been sustained or had increased during infusion of the drug Sodium, potassium, and chloride output tended to return toward control levels or continued to fall at this time Bicarbonate excretion typically diminished, usually in association with the decline in urine flow, and the urine became more acid Titratable acid, ammonium, and phosphate excretion did not change significantly

TABLE II. THE EFFECT OF L-NOREPINEPHRINE ON ACID-BASE EQUILIBRIUM

SUBJECT SEX AGE S.A.	PROCEDURE	B.P. (mM./Hg)	P.R.	PLASMA						
				Na (mEq./L.)	K (mEq./L.)	Cl (mEq./L.)	PO_4 (mM./L.)	HCO_3 (mM./L.)	pCO_2 (mM./Hg)	pH (units)
F.G.	Control	115/68	74	137	3.9	113	1.1	21.2	37.5	7.35
F 36 1.59	Infusion	154/82	72	137	3.6	109	0.8	23.3	39.9	7.37
	Recovery	130/70	--	137	3.1	111	0.8	24.2	43.9	7.41
E.P.	Control	100/62	60	133	3.9	110	1.2	24.6	43.5	7.39
F 37 1.62	Infusion	152/75	54	135	3.3	103	1.1	24.8	42.2	7.41
	Recovery	90/50	70	139	3.5	106	1.0	27.6	43.5	7.43
J.R.	Control	110/70	78	138	3.7	108	1.1	25.3	41.2	7.44
M 58 1.69	Infusion	152/80	72	--	3.6	108	1.2	25.6	44.2	7.40
	Recovery	110/60	--	141	--	110	0.5	26.7	36.9	7.47

See Table I for key to abbreviations.

*All values are avergaes of two or more determinations except for the recovery period. A continuous infusion of 2.25 per cent sodium bicarbonate was administered throughout the study. L-norepinephrine was administered during the "infusion" period. Subjects F. G. and E. P. had received 8 Gm. of ammonium chloride on the day prior to the study.

Ammonium chloride was administered in three subjects in order (1) to increase titratable acid and ammonium output to levels at which changes during l-norepinephrine administration would be more easily interpretable and (2) to assure an adequate excretion of cation. The dosages employed produced a slight reduction in the pH of plasma in two subjects (E. T. and A. M.) but were insufficient to produce excessive alteration in urinary composition (Table I). The changes induced by l-norepinephrine under these circumstances closely resembled those observed in subjects who did not receive ammonium chloride (Table I). Titratable acid and ammonium output were not affected significantly and sodium, potassium, and chloride output diminished abruptly.

In an attempt to separate the alterations in urine composition from the changes in plasma acid-base composition induced by l-norepinephrine an infusion of 2.25 per cent sodium bicarbonate was administered intravenously to three subjects at a rate sufficient to prevent the usual fall in plasma bicarbonate content elicited by l-norepinephrine. The results of this study are presented in Table II. Plasma bicarbonate concentration increased progressively throughout the study in each instance. Other plasma constituents were not affected significantly during the administration of sodium bicarbonate except for a tendency for the pH to increase and for sodium concentrations to rise and chloride concentrations to fall. The typical changes in urine composition elicited by l-norepinephrine occurred in these subjects. Sodium, potassium, and chloride output fell and no consistent change in urinary pH or ammonia or titratable acid excretion occurred.

Response to Ephedrine.—Ephedrine, which resembles the adrenal medullary hormones chemically but which differs in its effects on renal hemodynamics and electrolyte excretion,[4] was administered to 3 subjects (Table III) in order to evaluate further its effects on renal electrolyte metabolism.

The bicarbonate content of plasma and pCO_2 remained constant in two subjects (D. T. and E. C.) during the pressor response to ephedrine. In the third

…AND URINE COMPOSITION DURING INFUSION OF 2.25 PER CENT SODIUM BICARBONATE*

GFR (ML/MIN)	URINE									
	V (ML/MIN)	pH (units)	Na (μEq/MIN)	K (μEq/MIN)	Cl (μEq/MIN)	PO_4 (μM/MIN)	HCO_3 (μM/MIN)	pCO_2 (mM/Hg)	T.A. (μEq/MIN)	NH_4 (μEq/MIN)
118	3.5	5.20	193.0	113.5	325.3	9.4	0.7	35.8	16.5	--
104	5.1	6.28	21.6	47.1	37.8	4.1	7.8	32.4	6.8	--
116	1.5	5.88	78.5	36.7	64.7	11.4	1.1	40.1	6.5	--
141	6.4	5.13	192.8	109.0	313.5	17.2	1.1	42.3	24.4	45.4
94	3.5	6.59	39.5	52.2	63.5	10.2	11.7	33.0	7.5	16.7
99	0.7	6.48	53.4	39.6	47.0	14.4	2.5	47.6	5.4	8.9
105	5.8	6.96	74.8	110.9	108.9	11.4	53.4	43.4	6.1	6.8
107	6.3	6.66	35.6	75.6	45.7	9.9	33.1	46.4	12.2	7.1
100	4.9	6.58	27.6	55.7	34.5	8.1	19.7	41.8	10.6	7.5

subject (J. S.) the bicarbonate content of plasma decreased and fell further after the drug was discontinued. The sustained elevation of arterial pressure at this time in J. S. and in E. C. suggests continued pressor and perhaps metabolic activity of ephedrine during the "recovery" period. The acidity of plasma was unaltered during the infusion of ephedrine and the plasma concentrations of sodium, potassium, chloride, and inorganic phosphate remained constant. The changes in plasma composition thus resembled those elicited by the adrenal medullary hormones, but were less consistent, an observation which might have been predicted from previous observations of the effects of this agent on ventilation[20, 21] and on carbohydrate metabolism.[22] Urinary sodium and chloride output increased in two subjects (D. T. and E. C.) in association with an increment in glomerular filtration rate and diminished in the third study in association with a fall in filtration. Sodium output increased more than filtration in subjects D. T. and E. C., suggesting diminished tubular reabsorption per unit of filtrate. The sodium inulin clearance ratio did not change in J. S. Potassium output fell progressively in all three studies. These results are in substantial agreement with previous observations[4] of the effects of ephedrine on electrolyte excretion except that changes in filtration were more prominent in the present study. No consistent effect was noted on urinary acidity and CO_2 content or on titratable acid, ammonium or inorganic phosphate excretion.

DISCUSSION

L-epinephrine increases transiently the depth and rate of respiration when administered in physiologic amounts to man and induces a mild respiratory alkalosis.[9] Later, lactic acid accumulates in extracellular fluid secondary to accelerated glycogenolysis[8] in muscle, and extracellular bicarbonate content remains reduced despite apparent correction of the respiratory alkalosis.* L-norepinephrine induces similar but somewhat less pronounced changes,[7, 26] although alterations in plasma lactic acid content are not a prominent feature

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*The plasma concentration of other organic acids may also increase secondary to muscular activity during overventilation.[3, 24] However, in the present study urinary organic acid excretion did not change.

TABLE III. THE EFFECT OF EPHEDRINE SULFATE

SUBJECT	SEX	AGE	S.A.	PROCEDURE	B.P. (mM./Hg)	P.R.	PLASMA						
							Na (mEq./L.)	K (mEq./L.)	Cl (mEq./L.)	PO$_4$ (mM./L.)	HCO$_3$ (mM./L.)	pCO$_2$ (mM./Hg)	pH (units)
E.C.	F	48	1.49	Control	116/78	76	135	4.0	109	0.9	25.1	38.5	7.44
				Infusion	169/90	74	135	3.4	108	0.7	24.8	34.6	7.45
				Recovery	160/82	72	137	3.2	109	0.8	24.8	38.9	7.44
J.S.	F	21	1.90	Control	120/86	68	131	4.3	105	1.0	24.1	41.5	7.39
				Infusion	169/88	85	135	4.2	104	0.8	23.4	40.5	7.37
				Recovery	153/89	88	135	3.7	102	0.8	22.1	38.2	7.38
D.T.	M	40	1.69	Control	124/70	70	142	3.3	107	0.7	26.2	36.5	7.48
				Infusion	156/70	80	143	3.8	108	0.7	26.9	36.5	7.48

See Table I for key to abbreviations.

*All values are the averages of two or more determinations. Ephedrine was administered intravenously during the "infusion" period. Subjects E. C. and J. S. had received 8 Gm. of ammonium chloride on the day prior to the study.

of the later phase of acid-base adjustments.[27] Explanation of the reduction in bicarbonate content at this time is not clear. Adjustment to this later and more prolonged displacement of acid-base balance is apparently made almost entirely by changes in ventilation. In the present study the calculated carbon dioxide tension of plasma usually fell and renal mechanisms for compensation were not apparent. Although tubular reabsorption of sodium and potassium increased at this time, as has been noted previously,[1-3] these changes in tubular activity appeared to be independent of the accompanying systemic metabolic alterations. The tubular response to l-norepinephrine was unmodified when, as a result of an infusion of sodium bicarbonate, plasma bicarbonate content increased rather than diminished and the plasma became more alkaline. Moreover, tubular exchange of hydrogen (as titratable acid) and ammonium ions was not accelerated during the infusion of norepinephrine, and sodium and potassium returned to the circulation largely as chloride salts, a transfer which, if prolonged, would have tended to perpetuate rather than alleviate the accompanying reduction in plasma bicarbonate content. However, in some subjects bicarbonate-obligated cation was reabsorbed in increased amounts as well, and displacement of acid-base equilibrium was thereby minimized. This may account in part for the absence of disturbances of acid-base balance in clinical circumstances, such as pheochromocytoma, in which prolonged or excessive adrenal medullary hormonal activity might be expected, although other corrective adjustments may also occur which limit not only this disturbance but the retention of electrolytes as well.[3]

The factors which determine the relative rates of chloride and bicarbonate reabsorption are obscure. The availability of anion did not appear to condition tubular activity, although in subjects who had received ammonium chloride the relative abundance of chloride in tubular urine, from which practically all bicarbonate-obligated cation was extracted, resulted in the reabsorption of cation almost entirely as chloride salts. In part, the reabsorption of bicarbonate-obligated cation appeared to be independent of the action of l-norepinephrine. In many instances changes in the excretion of bicarbonate paralleled changes on Acid Base Equilibrium and Urine Composition*

GFR (ML / MIN)	URINE V (ML / MIN)	pH (units)	Na (μEq/ MIN)	K (μEq/ MIN)	Cl (μEq/ MIN)	PO_4 (μM/ MIN)	HCO_3 (μM/ MIN)	pCO_2 (mM / Hg)	T A (μEq/ MIN)	NH_4 (μEq/ MIN)
108	3.8	5.17	125.6	70.5	177.6	17.1	0.7	43.0	25.5	27.0
131	8.8	5.51	186.4	52.8	217.5	17.4	3.1	44.0	28.8	40.9
129	6.8	5.29	141.0	39.8	163.0	17.0	1.3	39.7	27.1	33.3
113	3.5	4.71	197.0	85.9	300.0	13.6	0.2	33.4	31.2	36.8
96	4.3	4.69	168.0	70.0	184.0	13.6	0.3	39.0	27.6	30.9
117	6.9	4.95	148.0	59.7	213.7	16.2	0.6	37.1	31.8	35.4
69	6.9	6.56	158.8	61.8	144.1	8.8	26.2	42.8	10.0	18.1
76	7.8	6.45	211.0	45.4	179.1	10.1	26.7	49.9	11.7	20.2

in urine volume and were independent of alterations in sodium and potassium output This phenomenon has been described previously[12, 19] and has been attributed to mixing of tubular urines of varying pH and buffer content[12]

The site within the nephron of the accelerated tubular transfer of electrolyte is unknown The data obtained in the present study are consistent with either proximal or distal tubular reabsorption, or both However, tubular exchange mechanisms at either site appeared to play little role, unless reabsorption of bicarbonate obligated cation is considered to occur in large part as a result of tubular cation exchange[28] The failure of urinary acidity to change consistently may be attributed to the relatively constant excretion of titratable acid, to the inconstant alteration in bicarbonate output, and to the buffering capacity of the urine

The mechanism of the electrolyte retaining activity of the adrenal medullary hormones was not clarified by the present study This response appears to be independent of systemic acid base adjustments and, as previous studies have demonstrated,[4] it is not mediated through secondary hormonal or neural pathways Moreover, the different urinary electrolyte patterns elicited by ephedrine and l norepinephrine, as demonstrated in this and previous investigations,[4] suggest that nonspecific pressor activity does not play a role There remains the possibility, not tested in the present study, that local intrarenal circulatory adjustments and reduction in renal blood flow to tubular tissue elicit the retention of sodium and potassium chloride and bicarbonate or that the adrenal medullary hormones condition this response directly through alteration of tubular cellular metabolism These views have been considered previously[4]

SUMMARY

L norepinephrine induced a reduction in plasma bicarbonate content with no change in pH when administered intravenously to normal human subjects Renal tubular reabsorption of sodium, potassium, and chloride and of bicarbonate obligated cation increased without consistent change in inorganic phosphate or ammonium excretion or in urinary pH or titratable acid excretion These changes were observed in subjects premedicated with ammonium chloride and were unmodified during an infusion of sodium bicarbonate which progressively

increased plasma bicarbonate content. These results suggest that alterations in urine formation elicited by l-norepinephrine were independent of the accompanying displacements of plasma acid-base balance. Ephedrine sulfate elicited similar but less constant changes in plasma acid-base composition, but tended to depress rather than augment tubular electrolyte reabsorption.

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USE OF METHYLENE BLUE AS AN INDICATOR FOR ARTERIAL DILUTION CURVES IN THE STUDY OF HEART DISEASE

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MULTIPLE recordings of indicator-dilution curves following selective injections of dye into various sites in the central circulation have become an indispensable part of diagnostic cardiac catheterization procedures performed in this laboratory.[1-4] Their greatest usefulness has been in the detection, localization, and quantitation of intracardiac shunts in patients with congenital cardiac malformations.[3, 5, 6] Recently, dye-dilution curves have also found application in the diagnosis of acquired heart disease.[7-9]

Frequently, in order to allow a definitive diagnosis to be made during diagnostic right or left heart catheterization, multiple arterial dilution curves are recorded following injection of dye into various sites in the heart and great vessels. Evans blue dye (T-1824), the indicator commonly used, has the disadvantage of causing bluish discoloration of the skin and mucous membranes when given in doses exceeding approximately 0.5 mg. per kilogram[10] which, in addition to the cosmetic effect limiting the number of curves which may be performed, interferes with determination of blood oxygen saturation by oximetry and with the clinical evaluation of cyanosis, as for example, after cardiac operations.

To avoid this bluish discoloration it was decided to reinvestigate the use of methylene blue as an indicator in dye-dilution curves, this substance having been previously used for this purpose[11, 12] and found not to produce bluish discoloration in this same dosage range.

Evans blue and methylene blue are both aniline dyes. Evans blue (T-1824), a triphenyl methane dye which has a molecular weight of 960.83, has been widely used for blood-volume studies.[13-15] It can be quantitatively determined in blood spectrophotometrically in accordance with the Lambert-Beer law. Methylene blue or tetramethyl-thionine chloride, which has a molecular weight approximately one third as great as that of T-1824 (373.90), on the other hand, cannot easily be accurately quantitated in this fashion, since it exhibits the so-called spectral anomaly in aqueous solution, that is, deviation from the Lambert-Beer law, for reasons which are as yet not completely explained but in which reversible polymerization of the dye molecules is generally agreed to play a dominant role.[16-18]

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Fig 1 compares the light transmission by aqueous solutions of both Evans blue* and methylene blue† dyes in equal concentrations of 5 mg per liter This graph also shows the sensitivity of the red photocell filter assembly, the actual detecting element of the earpiece or cuvette oximeter, for changes in the amount of oxyhemoglobin and reduced hemoglobin or for changes in dye concentration Since the peak sensitivity of the red photocell filter assembly is at approximately 640 mμ, it is apparent that methylene blue is photometrically *superior to Evans blue as an indicator for detection by oximetry* In this regard, it should be remembered that in similar concentrations of these dyes there are approximately three times as many molecules of methylene blue as of Evans blue Using the values for optical density obtained spectrophotometrically, the molecular extinction coefficients (Emol) for aqueous solutions (5 mg per liter) of Evans blue and methylene blue were calculated to be 40,000 and 60,000, respectively, at a wave length of 640 mμ

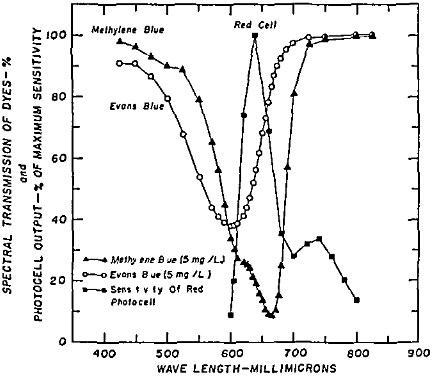

Fig 1—Comparison of light transmission of vans blue
and methylene blue dyes and the relative spectral assembly
of the oximeter Note that at the peak sensitivi methylene
blue solution absorbs more light than Evans blue r cent of
the light of this wave length as compared to 55 blue

METHODS

Dilution patterns were recorded simultaneously by earpiece and cuvette oximeters‡ following the sudden single injection of indicator using a technique previously described[1] Successive injections into the same site in the central circulation of equal doses of both methylene blue and Evans blue dyes 3 3 to 21 1 minutes (average 5 7) apart were performed in 12 normal persons and in 28 patients with heart disease At times the recording of the methylene blue curve preceded that of the T 1824 curve, while at other times the order was reversed The dosage of dye ranged from 0 1 to 0 3 mg per kilogram (mean 0 2 mg per kilogram) To minimize interference in recording of the curves due to fluctuations in arterial blood oxygen saturation (reduced hemoglobin absorbs light of the same wave length as the dyes), most of the dilution curves were recorded with the normal subjects

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‡Manufactured by the Waters Corporation Rochester Minn

amounting to only 4.3 per cent (1.4 to 7.0) of their peak deflection. This difference in the concentrations of the two dyes in arterial blood was even more striking at a time 30 seconds after the peak concentration, when the methylene blue deflection had fallen to only 2.9 per cent (0.9 to 4.8) of peak deflection while the deflection produced by Evans blue still comprised 16.0 per cent (10.4 to 21.0) of its earlier peak deflection, a value which in the case of Evans blue is related to the blood volume of the individuals studied[1] (Table I and Fig. 3). This difference in concentration in arterial blood of the two dyes could indicate the occurrence of one of three possibilities: namely, that methylene blue is rapidly and completely reduced in the blood stream to its colorless leuko form, that the dye is lost from the blood stream to the tissues on its first passage through the circulation, or that both of these events are taking place simultaneously.

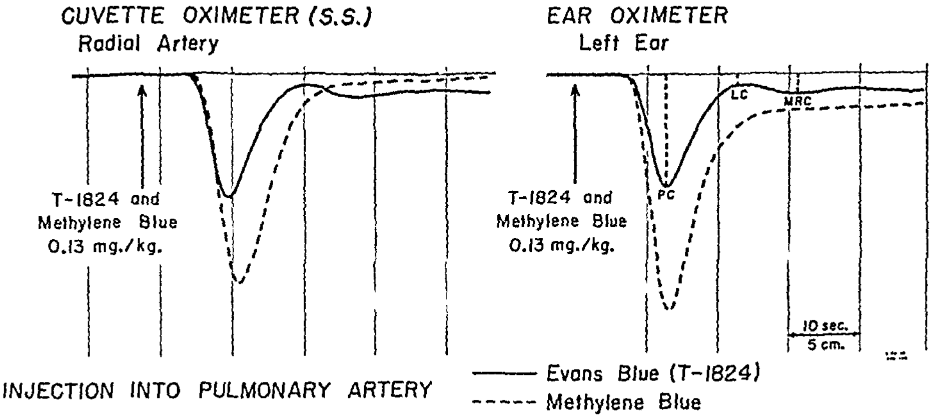

Fig. 3.—Arterial dilution curves recorded simultaneously by cuvette and ear oximeters following successive injections of Evans blue (solid lines) and methylene blue (dotted lines) dyes superimposed to facilitate comparison of their contours. The subject was a healthy man, 30 years of age. On the left are shown the curves recorded directly in arterial blood and on the right the ear oximeter curves. The Evans blue curves recorded directly in the blood and at the ears are similar, showing the usual pattern with a definite systemic recirculation peak and a [illegible] which is related to the blood volume. In the methylene bl[illegible]ater peak deflections and absent systemic recirculation peaks, [illegible] the dye to almost zero concentration in arterial blood within seconds after injection, while the earpiece curve at this same time still shows the presence of a considerable amount of the dye in the ear (see text).

Evidence that methylene blue rapidly passes from blood to tissue was obtained from the dilution curves simultaneously recorded at the ears (right panel of Fig. 3). While the peak deflection of the ear oximeter curves recorded after injection of methylene blue again was greater than that after injection of T-1824 (mean value 2.1 times as great; range: 1.4 to 2.6), the amount of dye detected at the ear for the remainder of the methylene blue curve compared much more closely to that for the Evans blue curve than was the case directly in arterial blood. Thirty seconds after the peak concentration, at a time when the methylene blue content detectable directly in arterial blood was reduced to only 2.9 per cent of the peak, the methylene blue deflection at the ears still amounted to 17.1 per cent of the peak deflection as compared to 19.1 per cent for T-1824 (Table II). With the knowledge obtained from the cuvette oximeter curves recorded directly in arterial blood that a relatively much smaller amount of methylene blue could be detected in the blood stream by this time, the conclusion that at least a portion of the methylene blue dye was entering the tissues (including ear tissue) during its first circulation seemed reasonable. Also supporting this view was the finding that in methylene blue curves recorded in normal subjects the systemic recirculation peak was either absent or markedly reduced and also the failure on several occasions to detect any recirculation of methylene blue dye at the ears or radial artery following the continuous, constant rate injection of relatively large doses of this dye (20 mg. per minute) into the descending aorta.[21] Both of the latter findings, however, could also be explained by rapid reduction of the dye in blood to its colorless form.

TABLE II. DILUTION PATTERNS AT THE EAR OF 12 NORMAL SUBJECTS AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF EVANS BLUE AND METHYLENE BLUE INTO THE PULMONARY ARTERY OR SUPERIOR VENA CAVA

	DEFLECTION AT			
	PC (CM.)	LC	MRC	30 SECONDS AFTER LC
		AS PER CENT OF PC		
Evans blue	6.1	12.3	22.1	19.1
Methylene blue	12.8	20.5	17.8	17.1
Mean difference	6.7	8.2	4.3	2.0
Range	-1.2 to 12.2	-1.8 to -17.1	-9.6 to 15.5	-17.7 to 14.4

PC = peak concentration. LC = least concentration. MRC = maximal recirculation concentration.

TABLE III. ABSENCE OF EVANS BLUE AND PRESENCE OF METHYLENE BLUE IN "BLOODLESS" EAR TISSUE IN 23 CASES APPROXIMATELY 2.5 MINUTES AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF THESE DYES

	OPTICAL DENSITY RATIO (D_R/D_{IR})		
	BEFORE INJECTION	1.4 TO 5.0 MINUTES AFTER INJECTION	DIFFERENCE
Evans blue	1.26 ± 0.02*	1.26 ± 0.02	0.00 ± 0.002
Methylene blue	1.25 ± 0.02	1.27 ± 0.02	0.02† ± 0.002

*The numbers following the ± signs are the standard errors of the mean values.
†Statistically significant: p value < 0.001.

Direct evidence for the rapid deposition of methylene blue in the tissues was obtained by spectrophotometric study of the light transmission of "bloodless" ear tissue. *Optical density ratios (D_R/D_{IR}) of the "bloodless" ear tissue,* calculated immediately before and at a mean time interval of 2.67 minutes (1.58 to 5.01 minutes) after injection of T-1824 into 23 healthy subjects and patients with heart disease, were not significantly different ($p < 0.9$). This confirms the previous findings of Connolly and Wood,[10] who also found no evidence of deposition of dye in the pinna of the ear or webs of the fingers immediately after injection of T-1824.

However, 2.36 minutes (1.42 to 4.01 minutes) after injection of an equal dose of methylene blue dye into the same 23 persons, the optical density ratios of the "bloodless" ear tissue were significantly increased over readings obtained before the injection ($p < 0.001$) (Table III). This corroborates the

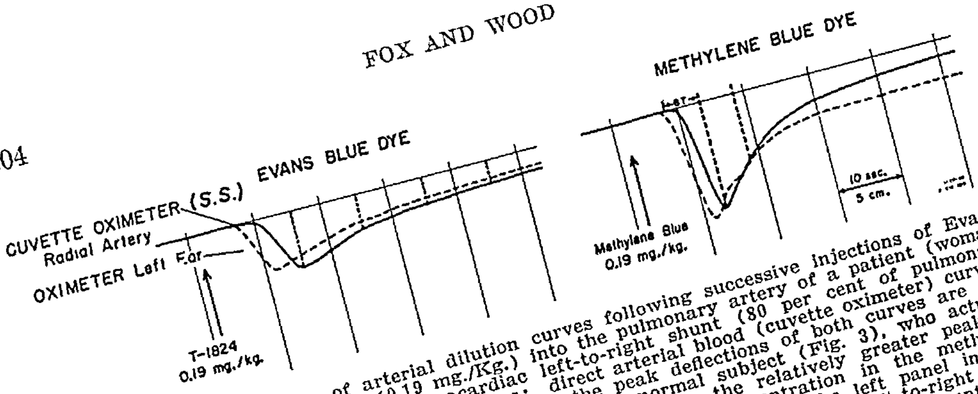

Fig. 4.—Comparison of arterial dilution curves following successive injections of Evans blue and methylene blue dyes (0.19 mg./Kg.) into the pulmonary artery of a patient (woman, 48 years of age) with a large intracardiac left-to-right shunt (80 per cent of pulmonary flow) via an atrial septal defect. Solid lines: direct arterial blood (cuvette oximeter) curves; dashed lines: ear oximeter curves. Note that the peak deflections of both curves are considerably smaller than in the curves recorded in the normal subject (Fig. 3), who actually received a proportionately smaller dose of dye. Note also the relatively greater peak deflection (PC) and the considerably faster decline in peak concentration in the methylene blue curve than in the Evans blue curve. Vertical dashed lines in the left panel indicate on the T-1824 curve the points at which deflections were measured in left-to-right shunt curves, that is, PC and the deflections at 10, 20, and 30 seconds after the peak concentration. Dashed lines in the right panel indicate points at which deflections were measured for calculation of the disappearance slope ratio. BT = build-up time on the cuvette curve.

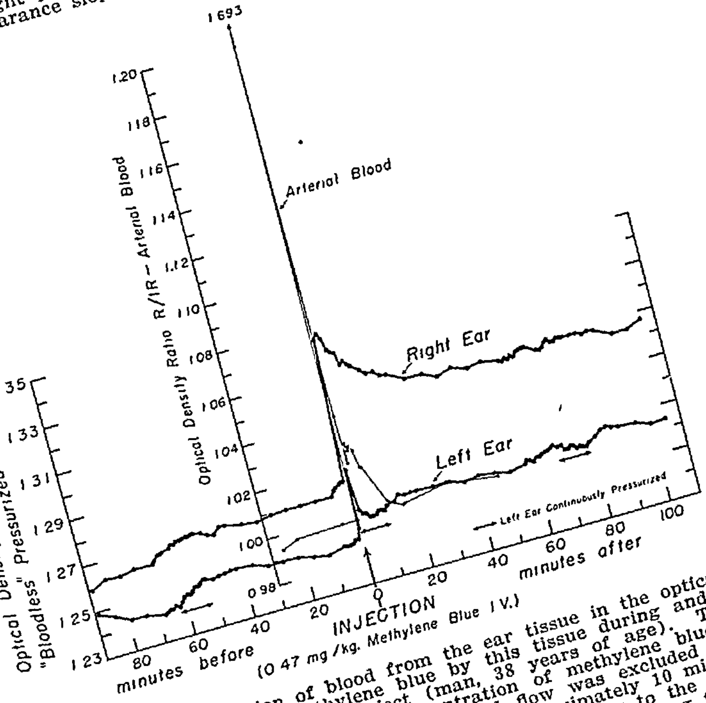

Fig. 5.—Effect of exclusion of blood from the ear tissue in the optical pathway of the ear oximeter on the pickup of methylene blue by this tissue during and after intravenous injection of the dye into a healthy subject (man, 38 years of age). The figure shows a comparison of the changes in apparent concentration of methylene blue in arterial blood and in "bloodless" (pressurized) ear tissue. Blood flow was excluded from the tissue in the optical path of the oximeter on the left ear for approximately 10 minutes beginning just prior to injection of the dye, while in the right ear blood flow to the tissue in the optical path of the oximeter was unimpeded during this period except during the 30-second periods required to measure the relative concentration of the dye in the pressurized ("bloodless") tissue of this ear. Note (1) the immediate high concentration (increase in optical density ratio) of methylene blue in arterial (radial artery) blood following injection and its subsequent rapid disappearance, (2) the immediate detection of a considerable quantity of methylene blue in the "bloodless" tissue of the right ear, and (3) that the apparent pickup of dye in the "bloodless" tissue of the left ear (from which blood was excluded during the period of high concentration in arterial blood) was much less than in the right ear. The initial peak of apparent concentration in the pressurized left ear, simultaneous with the peak concentration in arterial blood, is due to the effect of reflected light from the tissue contiguous to the pressurized area from which blood flow could not be excluded. Similarly, the small persisting increment in the optical density of the left ear tissue following the dye injection is probably produced in the same manner by the dye taken up by the contiguous (nonpressurized) tissues.

suggestion from the simultaneous cuvette and earpiece oximeter dye dilution curves indicating that the methylene blue dye was rapidly leaving the blood stream and at least a portion of it was being deposited in the tissues

To further confirm this observation a series of experiments was carried out in which dilution curves of methylene blue were recorded simultaneously in radial artery blood and at both ears, the blood, however, was excluded from the optical path of one earpiece during the dilution curve by inflating its pressure capsule to 250 mm of mercury When this was done the quantity of methylene blue subsequently detectable in the ear tissue from which blood was excluded during the dilution curve was strikingly less than in the ear tissue with unimpeded blood flow during and immediately after injection of methylene blue (Fig 5)

To study transformation of the spectral transmission of the dye in blood in vitro, successive sudden injections of Evans blue and methylene blue dye were made into oxygenated fresh human blood (heparinized) contained in a moving Waring blendor from which the blood was being continuously sampled through a cuvette oximeter Comparison of the resultant curves of dilution (Fig 6) shows an apparent disappearance of the methylene blue dye in the blood, which occurred at a progressively slower rate after initial mixing of the dye and blood This decrease in spectral absorption by methylene blue in blood may be due to the movement of dye into the cells and its intracellular reduction to the colorless leuko form

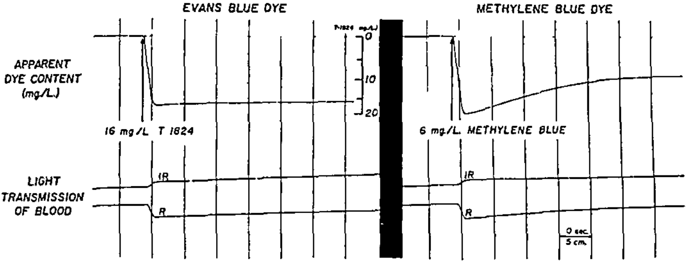

Fig 6—Original recording of apparent char s blue and
methylene blue immediately after mixing of dye , Note that
after a small overshoot (not instrumental) the ns blue in
the mixture remains stable In contrast a rapid lue occurs
apparently due to decolorization of the dye in bloc of methy
lene blue in the mixture decreased in 2 minutes to value in 1
remained approximately stable at this value for the 0 minutes)

B Patients With Left to right Intracardiac Shunts—Decrease in the magnitude of the peak deflection and prolongation of the disappearance slope associated with an absent systemic recirculation peak in T 1824 arterial dilution curves have become recognized as the hallmarks of the presence of a left to right shunt[6] (Fig 4) These findings have also been noted in dilution curves recorded in patients with severe valvular insufficiency[7 9] When compared to the mean peak deflections obtained in the normal subjects with the two dyes, the diminution in magnitude of the peak deflections in 10 patients with left-to-right shunts was similar in the methylene blue and T-1824 curves, the mean peak concentrations amounting to 66.1 and 63.5 per cent, respectively, of the mean values for peak concentration obtained in the normal subjects. The degree of prolongation of the disappearance slope, however, was considerably less in the curves recorded after injection of methylene blue than after injection of Evans blue. Comparison of the deflections detected directly in the arterial blood 10, 20, and 30 seconds after the peak deflection in successive methylene blue and Evans blue curves in these 10 patients with left-to-right shunts revealed deflections amounting to 51, 41, and 34 per cent of peak deflection in the Evans blue curves at these times compared to concentrations of 31, 16, and 11 per cent of peak deflection in the curves recorded following injection of methylene blue (Table IV). These figures, however, do indicate the presence also of definite prolongation of the disappearance slope of the methylene blue curves in patients with left-to-right shunts compared to curves obtained in normals in whom at similar times, dye concentrations were approximately one-fourth of these values (Table I). Gross inspection of the curves revealed no difference in the behavior of methylene blue as an indicator when the patients were breathing air or oxygen.

TABLE IV. DILUTION PATTERNS AT THE RADIAL ARTERY OF 10 PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS AFTER SUCCESSIVE INJECTIONS OF EQUAL DOSES OF EVANS BLUE AND METHYLENE BLUE INTO THE PULMONARY ARTERY OR SUPERIOR VENA CAVA

		10 SECONDS AFTER PC	20 SECONDS AFTER PC	30 SECONDS AFTER PC
	PC (CM.)	AS PER CENT OF PC		
Evans blue	5.2	50.6	40.6	34.0
Methylene blue	10.1	31.0	16.4	10.9
Mean difference	−4.9	19.6	24.2	23.1
Range	−11.4 to 0.3	1.4 to 28.8	12.5 to 41.3	11.4 to 41.2

PC = peak concentration.

TABLE V. COMPARISON OF DISAPPEARANCE SLOPE RATIOS CALCULATED FROM SUCCESSIVE EVANS BLUE AND METHYLENE BLUE ARTERIAL DILUTION CURVES IN 12 NORMAL SUBJECTS, IN FOUR PATIENTS WITH STENOTIC VALVULAR HEART DISEASE AND IN 12 PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS

	DISAPPEARANCE SLOPE RATIO IN:		
	NORMAL SUBJECTS	PATIENTS WITH STENOTIC VALVULAR HEART DISEASE	PATIENTS WITH INTRACARDIAC LEFT-TO-RIGHT SHUNTS
Evans blue	0.30* (0.21–0.38)†	0.32 (0.25–0.40)	0.65 (0.44–0.88)
Methylene blue	0.26 (0.16–0.34)	0.26 (0.15–0.37)	0.50 (0.35–0.72)

*Mean value.
†Range.

To quantitate the disproportionate prolongation of the disappearance slope caused by a left-to-right shunt, the so-called disappearance slope ratio was devised. This ratio is calculated by dividing the deflection occurring at a time equal to the build-up time (BT)[20] after the peak deflection by the peak deflection (PC): Disappearance slope ratio = (Deflection BT seconds after PC) ÷ PC (Fig. 4, right panel).

In Fig. 7 the disappearance slope ratios for the successive Evans blue and methylene blue curves recorded at the radial artery are compared with the magnitudes of the left to right shunts measured in 12 patients during the same cardiac catheterization procedure. A fairly good correlation between the size of the left to right shunt and the disappearance slope ratio is evident both for the Evans blue and for the methylene blue arterial dilution curves. The mean values and ranges for the disappearance slope ratios determined from the successive dilution curves recorded in normal subjects and in the cardiac patients with and without left to right shunts are given in Table V. Patients with valvular heart disease in whom the presence of valvular insufficiency was found at operation or necropsy or in whom there was a strong clinical suspicion of the presence of valvular insufficiency were excluded. As is evident both from Table V and from Fig. 7, there was no overlap of normals and left to right shunt patients when this ratio was calculated either from the T 1824 curve or from the associated methylene blue curve. As expected, owing to the rapid loss of methylene blue from blood, ratios calculated from methylene blue curves were systematically smaller than those for Evans blue.

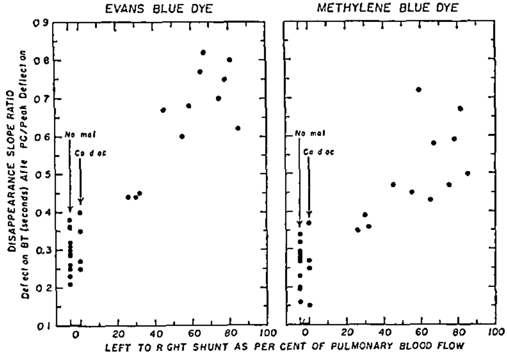

Fig. 7.—Relation of left to right shunt to disappearance slope ratio calculated from successive Evans blue and methylene blue dilution curves recorded at the radial artery in 12 patients with intracardiac left to right shunts. Note the fairly good correlation between the size of the left to right shunt (measured at cardiac catheterization) and the disappearance slope ratio of both the Evans blue (left panel) and methylene blue (right panel) dilution curves. The disappearance slope ratios obtained in 12 normal subjects and 4 patients with stenotic valvular heart disease (in whom both an intracardiac shunt and significant valvular insufficiency had been excluded) are plotted along the ordinate.

C. Patients Without Intracardiac Shunts.—In the nine cardiac patients without significant intracardiac shunts studied, comparison of the successive methylene blue and Evans blue arterial dilution curves yielded results which would be expected on the basis of the foregoing data. In 4 patients who had severe mitral or aortic stenosis (without significant insufficiency) the T 1824 curves were of the typical so called failure type, that is, the curves were shallow and all of the time components were markedly but symmetrically prolonged

(Fig. 8). Accompanying methylene blue curves revealed peak concentrations which were 1.9 (1.4 to 2.5) times as great as those after T-1824 and similarly displayed a prolongation of all of the time components. The disappearance slope of these curves, however, again was proportionately less prolonged than in the associated Evans blue curves, as was the case in the normal subjects and in patients with left-to-right shunts.

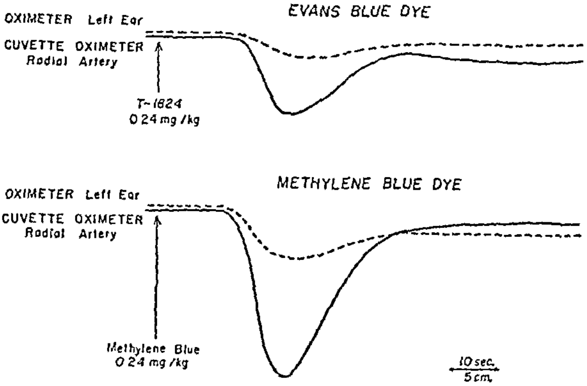

Fig. 8.—Comparison of arterial dilution curves following successive injections of Evans blue and methylene blue dyes into the pulmonary artery of a patient with stenotic valvular heart disease without an intracardiac shunt (man, 36 years of age; aortic stenosis). Note the *prolongation of all of the time components of these curves, especially the* prolonged systemic recirculation time of 52 seconds (normal: approximately 21 seconds). The systemic recirculation peak is evident only on the T-1824 curve recorded at the radial artery. The differences in amplitudes of the curves recorded at the ear and at the radial artery are due to the low sensitivity of the earpiece to dye in arterial blood which is encountered occasionally and occurred in this particular patient.

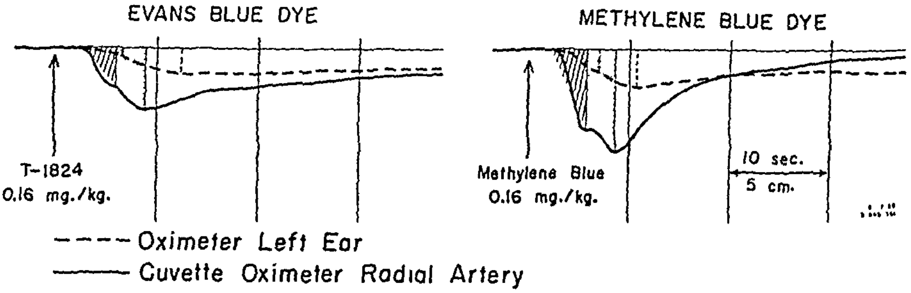

Fig. 9.—Comparison of arterial dilution curves recorded following successive injections of Evans blue and methylene blue dyes into the superior vena cava of a patient with an intracardiac right-to-left shunt (girl, 5 years of age; ventricular septal defect with pulmonary hypertension). The right-to-left shunt pattern consists of an early appearance of dye with a double hump contour, the first hump representing dyed blood which has passed through the defect directly into the systemic circulation to the sampling site and the second hump representing dyed blood which has followed the normal longer pathway through the lungs. The magnitude of the right-to-left shunt is determined by dividing the area of the first or shunt triangle (slanted lines) by the sum of the areas of the two triangles shown.[5] Note the proportionately greater area of the initial triangle in the methylene blue curve, which calculated out to represent a 40 per cent right-to-left shunt compared to a 25 per cent shunt calculated from the Evans blue curve. This difference is due to loss of methylene blue in the lungs coupled with rapid decolorization in the blood (see text). Note also the decreased amplitude and relatively poor definition of the curves recorded at the ear; this is sometimes seen, especially in infants.[4]

D. Patients With Right-to-left Intracardiac Shunts.—In 9 patients with congenital heart disease in whom the presence of an intracardiac right-to-left shunt was detected during cardiac catheterization by means of dye-dilution curves and, in some of the cases, also from the arterial blood oxygen saturation data, successive methylene blue and T 1824 curves were recorded at a mean time interval of 4.0 minutes (range 3.1 to 4.8 minutes). In 3 of the patients the veno arterial shunt was located at the atrial level and in the remaining 6 it was at the ventricular level.

Analysis of these curves was confined to a comparison of the magnitude of the calculated right to left shunts using the formula of Swan and associates[4] (Fig. 9). A similar percentage shunt would be expected if the areas of the primary and secondary deflections in curves recorded following the injection of each of these dyes maintained a similar proportion to each other.

From Table VI it can be seen that in the patients with moderately sized right to left shunts, all of whom had an interventricular communication, methylene blue curves tended to exaggerate the magnitude of the shunt (see also Fig. 9). In the 4 patients with atrial septal defect, all with small right to left shunts, the shunts calculated from the methylene blue and Evans blue dye curves were not significantly different.

TABLE VI. COMPARISON OF RIGHT TO LEFT SHUNTS CALCULATED FROM SUCCESSIVE DYE DILUTION CURVES USING EVANS AND METHYLENE BLUE

RIGHT TO LEFT SHUNT AS PER CENT OF SYSTEMIC FLOW IN					
FOUR PATIENTS WITH ATRIAL SEPTAL DEFECTS			FIVE PATIENTS WITH VENTRICULAR SEPTAL DEFECTS		
EVANS BLUE	METHYLENE BLUE	DIFFERENCE	EVANS BLUE	METHYLENE BLUE	DIFFERENCE
10	10	0	33	36	–3
			32	32	0
8	9	–1	29*	39	–10
10	11	–1	23	41	–18
			39	42	–3
			28*	50	–22
5	3	2	23	45	–22
			23*	31	–8

*Patient breathing room air; all other curves recorded with patient breathing 100 per cent oxygen.

The initial deflection of dilution curves recorded in the presence of a right to left shunt (Fig. 9) represents dyed blood which passes through the intracardiac defect directly to the systemic arteries and sampling site without traversing a capillary bed, while the second deflection represents dyed blood which has passed in the normal fashion through the capillary bed of the lungs. Therefore, if a significant amount of methylene blue disappeared from the blood stream during passage through the lungs a discrepancy in the estimate of veno arterial shunts calculated from methylene blue and Evans blue dye curves would be expected.

The technique of simultaneous right and left heart catheterization[22] afforded an opportunity of obtaining direct evidence with regard to possible loss of methylene blue dye in the lungs. Dilution curves were recorded simultaneously at the radial artery and at the ear following successive injections of equal doses of T 1824 and methylene blue into the left atrium and the pulmonary artery. Dye injected into the pulmonary artery must traverse the pulmonary capillary bed, while dye injected into the left atrium reaches the sampling site without passage through lung tissue. Also because of the longer distance to the sampling site, dye injected into the pulmonary artery remains in contact with the blood for a longer period than that injected into the left atrium, again giving a greater opportunity for disappearance of dye in the blood proper, as illustrated in Fig. 6. By comparing the ratio of the peak deflections recorded at the radial artery following the injections of identical doses of T-1824 (which is known to remain in the blood stream for a considerable period[10]) into the pulmonary artery and into the left atrium with the ratio obtained following the injection of methylene blue dye into these sites, an estimate of the average amount of dye disappearing during passage through the lungs is possible. In 5 such experiments, the amount of methylene blue dye "lost" in the lungs, as estimated by the foregoing method, was found to be 13 per cent (range: 1 per cent to 40 per cent).

DISCUSSION

In substituting methylene blue for Evans blue as indicator in arterial dilution curves an immediately apparent disadvantage is the inability to use methylene blue curves for cardiac output determinations, since this dye cannot easily be quantitated spectrophotometrically in blood. Methylene blue, however, finds considerable use as an indicator in diagnostic cardiac catheterization. In patients with left-to-right shunts it has been possible to estimate grossly the magnitude of the shunt from the Evans blue dilution curves.[6] Reorientation of the criteria used in this method of estimating the magnitude of left-to-right shunts is necessary when methylene blue is used, because of the (apparently) lesser degree of prolongation of the disappearance slope in the methylene blue curves. When this is done, a semiquantitative estimation of the magnitude of the left-to-right shunt is possible from the methylene blue curves of apparently the same degree of accuracy as when Evans blue is used. With regard to the detection of left-to-right shunts by means of dye curves it must be remembered that the systemic recirculation peak may be absent in methylene blue dilution curves recorded in normal subjects and that this deficiency cannot be regarded as an abnormal finding.

Because, milligram for milligram, it produces twice the deflection of T-1824, methylene blue has an advantage over T-1824 in the detection and localization of right-to-left shunts.

Another important function which methylene blue serves in the diagnostic applications of indicator-dilution curves is to permit the repetition of dilution curves, unsatisfactory for technical or other reasons, without fear of gross discoloration of the patient's skin, as would be the case were Evans blue the only available indicator.

Indigo carmine, which has recently been used in the performance of arterial dilution curves,[23] offers the possibility of overcoming some of the disadvantages of methylene blue. Because it is not lost from the blood stream before one circulation is completed, its dilution patterns can be used for determination of cardiac output, and for this same reason they resemble closely curves recorded following injection of T 1824 Its relatively low spectral absorption in the region of 640 mμ requires the use of comparatively large doses of this substance for the satisfactory recording of arterial dilution curves by means of oximetry However, this dosage does not produce appreciable discoloration of the skin[24]

SUMMARY

The contours of dilution curves recorded simultaneously at the radial artery and at the ears following successive injections of equal doses of Evans blue and methylene blue were studied in 12 healthy subjects, 28 patients with congenital heart disease, and 9 patients with acquired heart disease

1 Methylene blue in doses required for diagnostic purposes does not produce discoloration of the skin, as may occur following use of Evans blue

2 In equal doses methylene blue produced a peak deflection which was approximately double that following injection of Evans blue, however, the systemic recirculation peak was diminished or absent

3 Methylene blue was found to be largely lost from the blood stream on its first circulation and could be detected in bloodless ear tissue within seconds after its injection

4 Determinations of dye content in bloodless (pressurized) ear tissue 25 minutes after intravenous injection of 0 2 (0 1 to 0 3) mg per kilogram of Evans blue and of methylene blue revealed appreciable quantities of methylene blue but no detectable Evans blue in the tissue at this time

5 In vitro experiments revealed rapid (approximately 50 per cent) diminution in spectral absorption within 2 minutes after the mixing of methylene blue dye and whole blood The apparent concentration of dye in the mixture then remained approximately stable for the duration of the observations (30 minutes)

6 Appreciable apparent loss of methylene blue from the blood stream during its first passage through the pulmonary circulation was demonstrated

7 In patients with left to right shunts a similar degree of correlation was demonstrated between the degree of disproportionate prolongation of the disappearance slope of Evans blue and methylene blue dilution curves and the magnitude of the shunt as determined by cardiac catheterization

8 Methylene blue can be used in a similar fashion to Evans blue dye for the detection and localization of right to left shunts Loss of methylene blue in the dye blood mixture traversing the lungs may result in an overestimation of the magnitude of right to left shunts when this indicator is used

9 It is concluded that methylene blue is a valuable adjunct to Evans blue for use as an indicator for recording of selective dye dilution curves during diagnostic cardiac catheterization

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THE METABOLIC EFFECTS OF PROGESTERONE IN MAN: DOSE RESPONSE RELATIONSHIPS

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SEVERAL aspects of the metabolic influence of administered progesterone in men and women have been described in recent publications by this group.[1-4] Confirming an isolated experiment performed by Abels and Dobriner,[5] it was shown that progesterone in a dosage of 50 to 100 mg. daily consistently induced a moderate increase in the net rate of protein catabolism. Kyle and Hess[6] have obtained similar results. This nitrogen dissipation probably results from a direct or indirect effect on several organs or tissues, since, with the exception of muscle, no single tissue could provide the amount of protein catabolized as a result of this treatment. The process is not mediated by the adrenals, the pituitary, or thyroid glands and does not require the presence of adrenal or pituitary secretions. Estrogen does not potentiate the response and, although anabolic itself, does not inhibit it.

Progesterone was also shown to possess a transient sodium and chloride dissipating influence in subjects with normal adrenal glands, and a more intense and protracted saluretic effect in desoxycorticosterone and cortisone-treated Addisonians. In one adrenal deficient patient progesterone did not affect sodium and chloride excretion when the adrenal insufficiency was treated with salt alone, but after cortisone and desoxycorticosterone had been given the usual intense salt loss was induced. It was accordingly suggested that this effect of progesterone was probably explained by the competitive inhibition at a renal level of adrenal salt retaining hormones.

The 50 and 100 mg. daily dosages of progesterone which were employed in the above studies are amounts which, judging from pregnanediol excretion studies, approximate the quantity of progesterone secreted during early pregnancy.[2] From the standpoint of gaining an appreciation of the importance of the metabolic effects of progesterone in human physiology it was also necessary to determine its influence at dosages comparable to the amounts secreted at other times. Accordingly the effects of 6.25 to 300 mg. per day have been studied in several subjects, thus covering the physiologic range from the progestational phase of the menstrual cycle to the last trimester of pregnancy.

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*American Cancer Society Clinical Fellow.

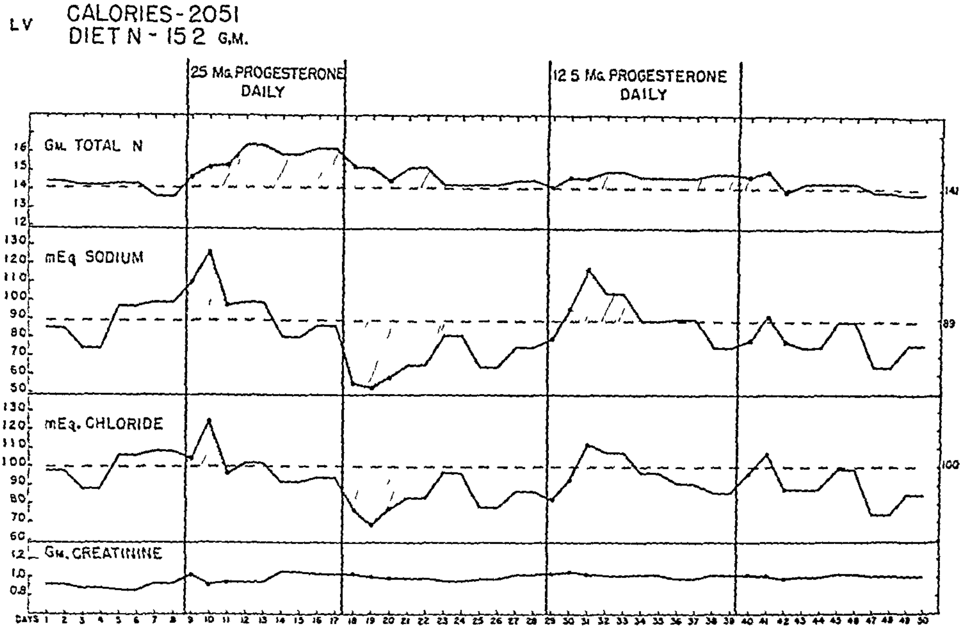

Fig. 1.—The effects of courses of 12.5 and 25 mg. of progesterone daily on the urinary excretion of total nitrogen, sodium, chloride, and creatinine in L.V., an arthritic man. Broken horizontal lines indicate the averages of pretreatment controls.

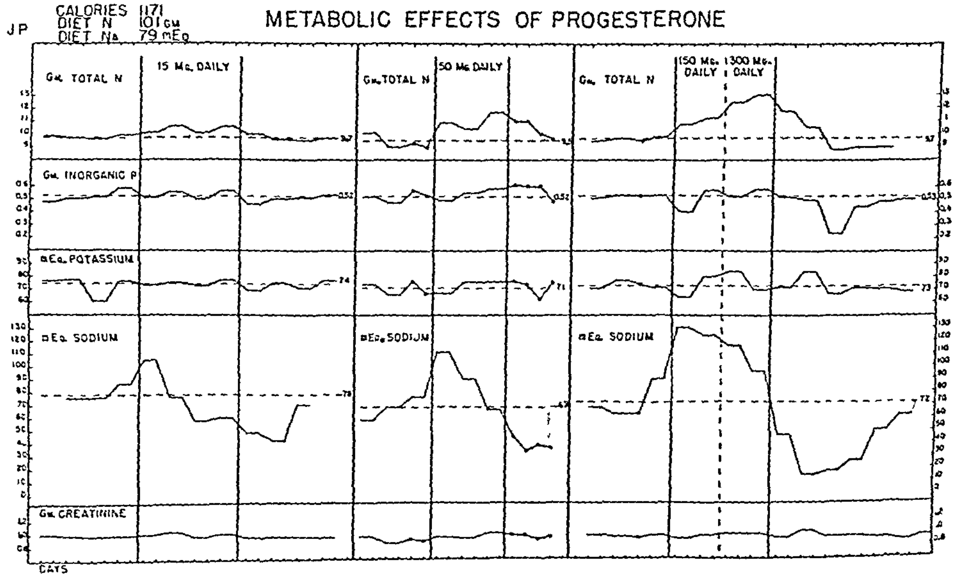

Fig. 2.—The effects of 15, 50, 150, and 300 mg. per day of progesterone on the urinary excretion of total nitrogen, inorganic phosphorus, potassium, sodium, and creatinine in J. P., the obese diabetic woman. Fasting and 4:30 P.M. postprandial blood sugars drawn during treatment varied from 112 to 168 mg. per 100 ml. and did not differ significantly from control levels. No glycosuria occurred at any time. Broken horizontal lines indicate the average of control values.

SUBJECTS

L V, a 60 year old man, was rather severely crippled by rheumatoid and degenerative arthritis *At the time these studies were performed he was afebrile and the rheumatoid* arthritis was inactive His basal metabolic rate (Mayo Foundation Standard) was +5 with basal calories of 1,460 per day

L S, a 53 year old postmenopausal woman, was chronically undernourished because of functional anorexia She weighed just 77 pounds The basal metabolic rate was −10 with basal calories of 820 For the sake of brevity detailed data from this experiment are not included, but will be referred to in the text

J P was a 56 year old postmenopausal woman suffering from diabetes, obesity, and arteriosclerotic heart disease When she was on a restricted diet no insulin was required, but when she ate freely, as was often her habit, she took as much as 80 units of insulin per day During the studies recorded here no insulin was required

R B was a 53 year old obese man with mild arteriosclerotic heart disease His metabolic rate was +7 with basal calories of 1,940 per day

E C was a postmenopausal woman with chronic neurodermatitis of the thighs Her metabolic rate was −15 with basal calories of 1,090 per day Detailed data are not presented, but are commented upon in the text

METHODS

All of the subjects resided in the metabolic unit of the hospital Physical activity varied little from day to day Dietary and fluid intake were maintained at a constant level in the usual fashion [7] The obese subjects were on moderate reducing programs, the others were fed maintenance or supramaintenance diets The progesterone, dissolved in sesame oil and 20 per cent benzyl benzoate, was administered intramuscularly in single daily injections for the smaller doses, but in 2 or 3 spaced injections for the 200 to 300 mg daily dosages Analyses were carried out on 24 or 48 hour urine pools, the completeness of collections being tested for by the performance of creatinine determinations As in the past a metabolic effect was assumed to have occurred when treatment was associated with a significant deviation from the base line values of the urinary constituent determined Complete balances were not performed The fecal excretion of nitrogen, inorganic phosphorus, sodium potassium, and chloride was assumed to vary insignificantly from day to day None of the patients had gastrointestinal disease Total urinary nitrogen, inorganic phosphorus and creatinine were determined as previously described [7] Sodium and potassium were measured by means of the Perkin Elmer flame photometer using a lithium internal standard Pregnanediol was determined by a modification of Stimmel's technique [8]

RESULTS AND DISCUSSION

In 3 subjects dosages of only 12 5 to 15 mg per day of progesterone evoked a catabolic response Although the effects were minimal, elevations in urinary nitrogen were consistent and systematically correlated with the time of progesterone administration In L V (Fig 1), the 12 5 mg course induced a nitrogen elevation which averaged 0 6 Gm daily In J P (Fig 2) and L S (data not shown), 15 mg daily of progesterone resulted in average increases of 0 7 and 0 6 Gm of nitrogen per day, respectively In L V, 6 25 mg of progesterone per day failed to affect urinary nitrogen excretion, although the characteristic rise in basal body temperature was observed It would thus appear that 12 5 mg per day of progesterone approaches the minimum dosage which could detectably enhance protein catabolism in man

Some gradation in the intensity of the catabolic response to progesterone was apparent as the dosage was increased to 25 and 50 mg. per day. In L. V. (Fig. 1) the daily nitrogen elevation averaged 1.7 Gm. during the 25 mg. daily course of progesterone, and in J. P. (Fig. 2) the nitrogen dissipation was increased to an average of 1.5 Gm. per day when 50 mg. was given. These subjects served as their own controls and the differences in response at the two dosage levels in each individual seem quite significant. Furthermore the 0.6 to 0.7 Gm. average elevation induced by 12.5 or 15 mg. of progesterone in these 3 experiments is distinctly less than the 1 to 2 Gm. increments observed as a result of the daily administration of 50 mg. per day of progesterone in over 20 published[2, 4] and unpublished experiments.

The elevation in urinary nitrogen tended to be maximal at a dosage of about 50 mg. per day. In some subjects, however, larger amounts of progesterone evoked a somewhat greater response. In a previously reported experiment[2] 100 mg. daily induced a nitrogen elevation of 1.7 Gm. per day in comparison with the 1.1 Gm. average increase which resulted from treatment with 50 mg. The average elevation induced by 50 mg. per day in J. P. (Fig. 2) was 1.5 Gm.; during the 4 days on 150 mg. the increase averaged just 1.4 Gm., but doubling the dosage in the succeeding 4 days was associated with a further increase to a level 3.0 Gm., higher than the control base line. On the other hand, in R. B. (Fig. 3) the nitrogen elevation induced by separate courses of 50, 200, and 300 mg. of progesterone per day were essentially the same, and in the postmenopausal woman, E. L. (data not shown), the nitrogen increments were also indistinguishable on these dosages. It may be concluded that even if these large quantities of progesterone should increase the catabolic process beyond that induced by 50 mg. per day, the urinary nitrogen elevation remains of the same order of magnitude, and the relatively extreme elevations which may be induced by large excesses of adrenal corticoids[9, 10] are not seen when as much as 300 mg. of progesterone is given each day. In considering this comparison, however, it should also be emphasized that when cortisone is given in dosages of 50 mg. per day or less, there is often no detectable catabolic response.

The influence of progesterone on urinary inorganic phosphorus and potassium in R. B. (Fig. 3) is unique in our experience. It has been stated[2] that when progesterone is given to individuals with normal adrenal glands the excretion of these constituents is not significantly affected. As a matter of fact, in about one-half of our experiments urinary potassium dropped approximately 7 meq. and inorganic phosphorus about 0.1 Gm. during the first two days of treatment. These shifts were more or less disregarded because they were trivial and inconsistent. In R. B. (Fig. 3), however, this prompt suppression in the excretion of both substances was conspicuous at all 3 dosages. Inorganic phosphorus dropped 0.24, 0.23, and 0.24 Gm. per day, and potassium 21, 12, and 14 meq. per day. The excretion of both then returned to the base line and rose above it in parallel fashion, returning to the control level several days after treatment had stopped. These shifts probably represent an unexplained exaggeration of the dip in urinary potassium and inorganic phosphorus seen in some other subjects (Fig. 2; 150 mg. dose). As such they may be assumed to reflect a metabolic change involving both phosphate and potassium.

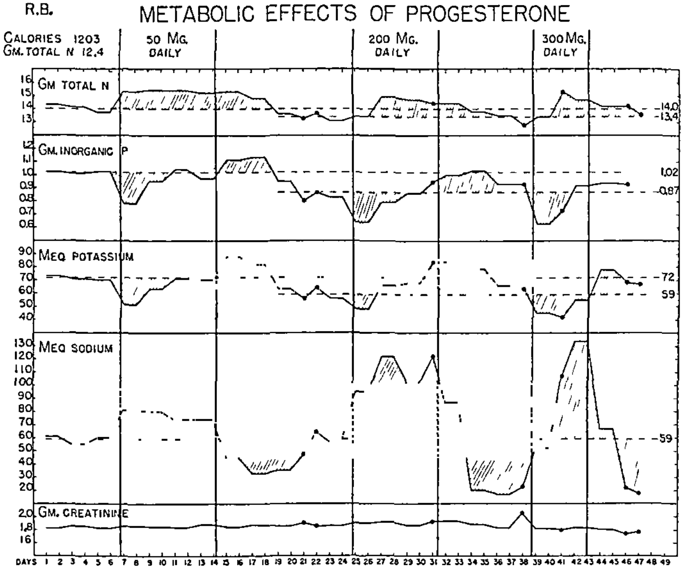

Fig. 3.—The effects of courses of 50, 200, and 300 mg of progesterone each day on the urinary excretion of total nitrogen, inorganic phosphorus, potassium, sodium and creatinine in R. B., the obese man. Broken horizontal lines indicate the averages of control values. Two control levels are indicated in the excretion of total nitrogen, inorganic phosphorus, and potassium because the base line of these constituents dropped after the first treatment.

The dissociation of these parallel shifts in potassium and phosphorus from the rising curves of urinary nitrogen excretion suggests that whatever metabolic process is involved, it is relatively independent of protein catabolism. In some circumstances, i.e., anabolic processes induced by androgen,[7] the urinary inorganic phosphorus and potassium excretion closely paralleled total nitrogen excretion, indicating their storage with protein tissue. In some Addisonians[2] in whom a rather intense catabolism was induced by progesterone the urinary excretion of inorganic phosphorus and potassium rose with the total nitrogen, presumably reflecting the acceleration of protein tissue dissolution. The sharp initial decline in the excretion of inorganic phosphorus and potassium which is dramatized in R. B. (Fig. 3) seems to point to some other influence of progesterone—perhaps an effect on carbohydrate metabolism. In this connection it should be noted that in the controlled diabetic subject J. P. (Fig. 2) progesterone did not induce glycosuria, and the fasting and postprandial blood sugars were not affected by treatment.

The intensity of the salt dissipating action of progesterone was very clearly enhanced by increasing the dosage of the hormone (Fig. 1). The calculated losses of sodium in L. V., J. P. (Fig. 2), and L. S. on 12.5 to 15 mg. of progesterone daily were 68, 46, and 22 meq., respectively. In L. V., the sodium loss on 25 mg. per day was 85 meq., and in J. P. and R. B. the losses during the 50 mg. treatment period were 132 and 138 meq., quantities representative of the usual losses at this dosage. The magnitude of the sodium losses was inevitably impressive and more enduring when 150 to 300 mg. of progesterone per day were given. In J. P. (Fig. 2) the 300 mg. dosage was begun after just 4 days on 150 mg. daily—at a time when the induced natriuresis had begun its characteristic decline. The 8-day loss, however, was 336 meq. In R. B. the loss on 200 mg. daily was 396 meq.; since the 300 mg. course was short and was started during the post-treatment phase of sodium retention, calculations would not be appropriate, but the peak excretion of sodium was higher than at any other time. Entirely similar results were obtained in E. C. (data not shown). Urinary chloride excretion (not shown in Figs. 2 and 3) closely followed that of sodium, but as previously noted[2] the losses were never so great.

As in other experiments in subjects with normal adrenals, immediately following the cessation of treatment with progesterone, urinary sodium excretion dropped abruptly and in the ensuing several days virtually all of the sodium which had been lost was recovered. Following the large doses of progesterone the intensity of the salt retaining impulse was remarkable, the excretion of urinary sodium dropping to levels of only 15 to 25 meq. per day. This contrasts with the treated Addisonians in whom salt loss of the same magnitude was not followed by a sharp retention. It has been suggested[2] that this suppression of urinary sodium may be the result of an increased secretion of adrenocortical salt retaining steroids.

It should be pointed out that in none of the many subjects in whom we have studied the influence of progesterone has there been evidence of preexisting salt and water retention. There is no reason to believe, however, that progesterone might not be saluretic in patients with edema. Leutcher and associates[11] have shown that patients with heart failure, nephrosis, etc., excrete an increased quantity of aldosterone suggesting that this adrenal hormone may play a role in the genesis and/or maintenance of such edema states. If, as has been indicated, the natriuretic influence of progesterone is due to the inhibition of the renal effects of salt retaining corticoids, it is conceivable that progesterone would promote the urinary excretion of salt in such conditions. If so, the post-treatment retention of sodium might be lessened by placing the patient on a low-salt intake.

Although the dosages studied ranged widely from 6.25 to 300 mg. daily, there is every indication that each of these quantities may be presented to the female organism under physiologic conditions. Calculations based on the fact that 10 to 20 per cent of administered progesterone is excreted as pregnanediol[2, 12, 13] indicate that as much as 25 mg. of progesterone may be secreted each day by the corpus luteum of the nonpregnant woman. This deduction is supported by the fact that in L. V. (Fig. 1) 3.3 mg. of pregnanediol per day were excreted during the 25 mg. dosage period and 2.2 mg. per day when 12.5 mg. was given. Both quantities are within the range of pregnanediol excreted during the luteal phase of the menstrual cycle. The 50 mg. dosage probably can be considered as approximating the quantity secreted in early pregnancy, and 200 to 300 mg. per day late pregnancy. J. P. (Fig. 2) excreted 62 mg. of pregnanediol per day when receiving 300 mg. of progesterone. It remains important to demonstrate that endogenous progesterone exerts similar effects, but these data suggest that progesterone might influence protein metabolism and sodium and chloride excretion in the pregnant and the normal menstruating woman.

SUMMARY AND CONCLUSION

1. Progesterone exerts a demonstrable catabolic effect in men and women when administered in dosages which approximate the levels of secretion of the hormone during the luteal phase of the menstrual cycle and early and late pregnancy. Although 6.25 mg. per day had no effect, 12.5 to 15 mg. induced a distinct elevation in urinary nitrogen excretion which averaged about 0.7 Gm. in 3 subjects. Doses of 25 to 50 mg. of progesterone evoked a greater response, the usual nitrogen elevation, averaging 1 to 2 Gm. per day. The intensity of the catabolic effect of progesterone tended to plateau at a dosage of 50 mg. per day. Larger quantities (100 to 300 mg. per day) did not regularly induce greater responses.

2. In one man urinary inorganic phosphorus and potassium dropped significantly during the first 2 or 3 days of progesterone treatment, followed by a return to the base line, and then an overshooting of control levels. A similar slight decline in potassium and inorganic phosphorus had been seen in approximately half of our other studies. Since urinary nitrogen excretion was rising at this time it was suggested that these shifts did not reflect the change in protein metabolism, but some other process.

3. In one mild, controlled diabetic progesterone did not affect the blood sugar level or induce glycosuria.

4. The intensity and duration of the natriuresis induced by progesterone was clearly related to the dosage. The effect was slight and transient at the low doses, but much greater and more enduring when the dosage reached 150 mg. per day. In all cases the salt loss was recovered during the posttreatment phase of retention.

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SEQUENTIAL ANALYSIS IN THERAPEUTIC RESEARCH

I. APPLICATION TO BINOMIAL DATA AND TO MEASURED DATA NORMALLY DISTRIBUTED (ONE-SIDED ALTERNATIVE)

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THERE is an increasing need for a rapid and reliable method of evaluating pharmacodynamic agents for their potential therapeutic effects. The points for emphasis in the design of the therapeutic experiments are adequate randomization in the double-blind technique, and the accurate estimation of the variation in the variables measured, including the variation due to uncontrollable extraneous factors as well as that due to placebo effects. Usually the design is developed to correspond with the statistical technique by which the results are to be evaluated; thus, the collection of data proceeds until a sizable number of control and test observations have been gathered before a statistical analysis is attempted. Using the sequential method of Wald,[1] however, analysis begins as soon as the data from the control observations are available and continues with each new test observation until a decision is reached. The decision is usually reached and then the testing may be stopped after substantially fewer observations than are required by any other statistical plan now known.[2]

Sequential analysis, originally developed by Wald and associates for quality control in industry, has been applied to biologic and clinical problems at a purely theoretic level[3, 4] and has recently been reported to determine the significance of differences in proportions observed in clinical prophylactic and therapeutic trials[5] (binomial distribution), and to determine the significance of differences in measured variables in various biologic systems in man in terms of the likelihood ratio or the probability ratio tests against a single alternative hypothesis.[6-8] The technique of sequential analysis requires the randomization of the order in which tests are carried out and exploits the tendency of such a distribution to equalize in the long run; i.e., if in tossing an unbiased coin several heads appear without tails, the chance for a tails on the next toss is still "50-50" but it is inevitable that eventually a run of tails will appear so as to ultimately equalize the distribution. Sequential analysis is particularly adaptable to the apraisal of an effect on a biologic system whose intrinsic variability has already been determined, but it requires that this solid base line of control data be available for comparison.

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The first study reported here concerns data that is distributed binomially, in this instance the presence or absence of vomiting following the exhibition of antiemetic agents. The second involves data in which actual measurements were made. The control data, from which the mean and standard deviation were computed, were normally distributed. In this instance the study was designed to measure the day-to-day changes in 24-hour urine volume observed during a control period and during a period of administration of a diuretic agent. Significant change in the measured variable (24-hour urine volume) during treatment occurred in one direction only.

METHODS

The details of the clinical experimental studies herein analyzed as well as the results of their analysis by more standard statistical methods have been reported elsewhere.[9, 10] Briefly, the first study was concerned with the antiemetic effects of 3 identically appearing capsules. The human volunteers on each occasion were given 1 capsule prior to the ingestion of an emetic dose of ipecac. The capsules contained either a placebo, 20 mg. of chlorpromazine (Drug A), or 20 mg. of an experimental antiemetic compound structurally related to chlorpromazine, SKF 4657* (Drug B). The second study involved the measurement of the diuretic effect of two pyrimidinedione compounds, aminometramide† and aminoiso-

Table I

WHEN P_0 (%) =	VALUE OF P_1 (%) AT 99% CONFIDENCE INTERVAL WHEN $\bar{n}$ =						
	10	12	16	20	30	60	100
0	(41)	(36)	(28)	(23)	(16)	(8)	(5)
1	43	37	30	25	18	10	7
2	44	38	31	27	20	12	9
3	46	40	33	28	22	14	10
4	47	42	35	30	23	16	12
5	48	43	36	31	25	17	13
6	49	45	37	33	26	18	15
7	51	46	39	34	28	20	16
8	52	47	41	36	29	21	17
9	53	49	42	37	31	23	19
10	54	50	44	39	32	24	20
15	60	56	49	45	38	31	26
20	65	61	55	51	44	37	32
25	69	66	60	56	50	42	38
30	74	70	64	61	55	47	43
35	77	74	69	66	60	52	48
40	81	78	73	70	64	57	53
45	84	81	77	74	69	62	58
50	87	85	81	78	73	67	63
55	90	88	84	82	78	72	68
60	92	91	88	85	82	76	72
65	94	93	91	88	85	80	77
70	96	95	93	91	88	84	81
75	98	97	95	94	92	88	85
80	99	98	97	96	94	91	89
85	(100)	99	99	98	96	95	93
90	(100)	(100)	(100)	99	98	97	96
95	(100)	(100)	(100)	(100)	99	99	99

( ) = Impossible in sequential binomial formulas. Included for interpolation purposes only.

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*Smith, Kline, and French, Research *Laboratories, Philadelphia, Pa.*; *2-chloro-10-[3'-(N-methylpiperzinyl)-propyl]-phenothiazine dimaleate.*

†Brand of aminometramide (1-allyl-3-ethyl-6-aminotetrahydropyrimidinedione): *Mictine.* G. D. Searle & Co., Chicago, Ill.

metradine,* and a placebo administered orally to human subjects with normal cardiovascular renal systems under controlled conditions. Both studies were conducted according to the double blind technique using a systematized randomization procedure.

The derivation of the sequential graphs was as follows.

Binomial Distribution.—P_0 = unacceptable proportion of therapeutic successes (in this case, the antiemetic responses observed in the placebo control group).

P_1 = acceptable proportion of therapeutic successes (in this case, the antiemetic responses required in the treatment group to be considered significantly better than the placebo control group at an arbitrarily selected 99 per cent confidence interval on the basis of a given number of observations. This value may be obtained from Table I when P_0 is known and the average number of observations to be made has been decided upon. In general, we have found an average of 10 or 12 observations desirable.)

As an alternative method, P_1 may be arbitrarily designated by the investigator as the desirable or acceptable proportion of therapeutic successes based upon his past experience and special knowledge in the field of investigation. The formulas for $\bar{n}\,P_0$ and $\bar{n}\,P_1$ which follow may then be solved and the average number of observations necessary for a decision will be determined. When P_1 is thus selected and is set too close to P_0 in value, the average number of observations necessary for a decision will become large, but will remain smaller in general than that required by other standard statistical techniques. We have found, however, that by designating the average number of observations as 10 or 12, and then selecting P_1 as described above, one generally obtains a clinically, as well as statistically, acceptable difference between P_0 and P_1. We prefer this technique for general use, as the number of observations needed for decision generally remains small. However, the choice between these two entirely different methods for selecting P_1 rests entirely with the investigators and the clinical and experimental strategies which they wish to employ.

The drawing of a sequential graph requires that there be plotted a lower line to form the margin of one area (in this instance, unacceptable or "reject") and an upper line to form the margin of another area (in this instance, acceptable or "accept"). Between these lines lies the zone of indecision. The lines are partially determined according to the desired values for alpha and beta.

α = the maximum risk or probability of accepting proportions of therapeutic successes equal to P_0 or less (we selected $\alpha = 0.01$).

β = the maximum risk or probability of rejecting proportions of therapeutic successes equal to P_1 or greater (we selected $\beta = 0.01$).

The following notation was used to facilitate the computation of the upper and lower lines[2]:

$$g_1 = \log \frac{P_1}{P_0}$$

$$g_2 = \log \frac{1 - P_0}{1 - P_1}$$

$$a = \log \frac{1 - \beta}{\alpha}$$

$$b = \log \frac{1 - \alpha}{\beta}$$

(Values for a and b may be obtained from tables in the publication by the Columbia University Statistical Research Group.[2])

Intercept for lower line $= h_0 = \dfrac{b}{g_1 + g_2}$

Intercept for upper line $= h_1 = \dfrac{a}{g_1 + g_2}$

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*Brand of aminoisometradine (1 methallyl 3 methyl-6 aminotetrahydropyrimidinedione). Rolicton. G. D. Searle & Co., Chicago, Ill.

vomiting. Drug A is rejected on the sequential graph on the first 8 tests and also by plotting separately the next 18 tests and the next 12 tests. Drug B is rejected on the first 6 tests and also by plotting separately the next 26 tests and the next 9 tests (theoretic average numbers of tests required here are 14 for acceptance and 13 for rejection). Thus, all of the conclusions reached by sequential analysis for both Drug A and Drug B, concerning their effectiveness against vomiting, are that neither agent is significantly better than the placebo. Ordinarily, one would stop the experiment with a test agent after the first decision is reached. Here, analysis of both drugs was continued using further observations until 3 decisions with each drug were reached, primarily to indicate the consistency of decision by this technique. Analysis of this data by more standard statistical techniques has been reported previously.[9]

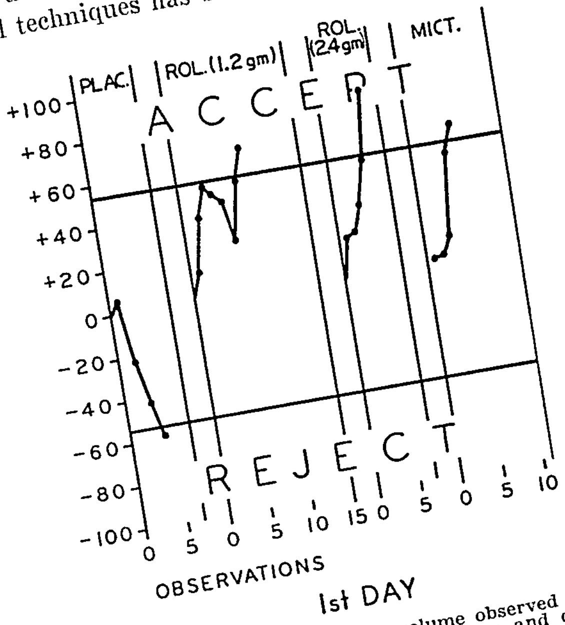

Fig. 2.—The results of the changes in urine volume observed during the first day of the administration of a placebo, of 1.2 and 2.4 Gm. of Rolicton and of 1.2 Gm. of Mictine compared to the control plotted sequentially and in summation fashion until an answer is obtained for each drug treatment group. The abscissa represents n (the number of observations) and the ordinate the Σ (X-S) (the sum of each change in 24-hour urine volume minus the slope observed on the first treatment day). Mictine and Rolicton, in both the small and large dose, are accepted on the first day as inducing an increase in urine volume significantly greater than that observed during the control period. The placebo is rejected on the first day as not inducing a significant change in urine volume compared to the control.

2. *Measured Data.*—In the experiments in which the diuretic activity of various compounds was measured in healthy subjects the urine volume measurements observed in the control observations were normally distributed. There were 23 subjects tested. The 24-hour urine volume was expressed as per cent of intake (output/intake × 100); the mean for each subject for the 3 control days was calculated and the daily variation from this mean was recorded not only for the 3 control days but also for each of the 3 treatment days that followed. Only the first treatment day is reported here. The standard deviation

of the control observations was 14 1 Solving the formula given previously under methods we find $m_1 = 17\ 3$ using $n_1 = 6$ Alpha and beta are again selected as 0 01 and solution of the appropriate formulas yield $h_0 = 52\ 84$, $h_1 = 52\ 84$, $S = 8\ 6$ Fig 2 presents the results plotted on the sequential graph thus constructed, from the first treatment day for each drug tested, including the placebo The Slope (S) was subtracted from each individual observation as was done in the derivation of this sequential graph (see methods) The placebo was rejected on the sequential graph as it failed to induce a change in the 24 hour urine volume which differed significantly from the control observations Aminoisometradine (Rolicton) was accepted on the first treatment day as having diuretic properties in both the dose of 1 2 Gm and 2 4 Gm per day, and aminometramide (Mictine) was accepted on the first treatment day at a dose of 1 2 Gm per day The number of tests required to make the above decisions was 4 for the placebo, 8 for the 1 2 Gm dose of aminoisometradine, 5 for the 2 4 Gm dose of aminoisometradine, and 5 for the 1 2 Gm dose of aminometramide The individual observations from this experiment have been reported previously in tabular form, together with analysis of the data by more standard statistical techniques[10]

DISCUSSION

*The technique of sequential analysis has been found convenient and re*liable when applied to clinical therapeutic experiments and yields a satisfactorily accurate decision regarding the efficacy of the agent tested with fewer observations than are required by more standard statistical techniques currently in use By plotting the result of each individual observation as it is made, it permits one to stop an experiment as soon as a decision is reached It is readily applicable to clinical data binomially distributed and to measured data normally distributed

Several deviations from the usual methods of applying sequential analysis to clinical data are described First in dealing with a binomial distribution, instead of arbitrarily selecting P_1 when P_0 is known, and then calculating the average number of observations ($\bar{n}\ P_1$) needed to establish statistical difference, we have chosen to arbitrarily fix n_1 at a desirable number and then to determine P_1 (from Table I) That the differences between P_0 and P_1 will also be of clinical significance is practically insured if n_1 is kept at 10 or 12 Second, in dealing with measured data the usual plans have most often selected m_1 as $2\ \sigma$ from m_0, or at another purely arbitrary distance from the known m_0 and then calculated the average number of observations ($\bar{n}\ m_1$) needed to demonstrate a significant difference We have chosen to arbitrarily fix n_1 (or $n\ m_1$) at a desirable number and then calculate m_1 by the method given In our experience, when m_1 is arrived at in this way it has always been acceptable as being significantly different clinically from m_0 Should it not be, however, one may widen the gap between m_0 and m_1 arbitrarily by raising the value of m_1 and then recalculating $n\ m_1$, or, should the difference seem clinically too great, the gap may be arbitrarily narrowed and $n\ m_1$ recalculated It is doubtful that either of these maneuvers shall prove necessary very often Finally, in the construction of the one-sided alternative sequential graph dealing with measured values we have chosen to routinely subtract the Slope (S) from each calculated value for the upper and lower lines and from each observation value to be plotted. This changes the appearance of the usual sequential graph so that it no longer has slanting upper and lower lines, but rather horizontal upper and lower lines which remain parallel to the abscissa and at right angles to the ordinate of the graph. This in no way alters the decisions reached, or their significance.

SUMMARY

A method is presented whereby the statistical technique of sequential analysis has been applied to clinical data binomially distributed as well as to measured data normally distributed. This technique permits the plotting of each individual test observation as it is made, and permits stopping the experiment when a decision is reached. Further, it generally requires fewer observations than other more standard statistical techniques currently in use. It should become a valuable aid for the evaluation of therapeutic agents.

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SEQUENTIAL ANALYSIS IN THERAPEUTIC RESEARCH

II Application to Measured Data Normally Distributed (Two-Sided Alternative)

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Earlier adaptations of sequential analysis to clinical data have allowed for a bidirectional alternative in data binomially distributed and for the detection of a significant change in measured variables in one direction, or the detection of a significant change in measured variables in either direction without indicating the direction of the change[1-9] The present report concerns the adaptation of sequential analysis to the study of the effects on various biologic systems in man of pharmacodynamic agents which may produce significant changes in measured variables in one direction or the other from the control observations, in order to indicate not only significant change but also the direction of that change (the two-sided alternative).

MATERIAL AND METHODS

Data from three separate clinical studies designed to measure the pharmacodynamic effects of certain therapeutic agents are described

The first study involved the effects of reserpine† and an anticholinergic gastric inhibitor (AP 396)‡ on gastric acidity Patients from the general medical wards served as subjects and comprised primarily those recently recovered from an acute illness without known gastrointestinal tract disease A few patients with past histories suggesting chronic duodenal ulcer and minor upper gastrointestinal tract symptoms were included. Following a 12 hour fast, 3 samples of gastric juice were collected at 15 to 30 minute intervals during a ½ to 1 hour control period Attempts were made during the collection of these and all subsequent samples to empty the stomach as completely as possible. After the control period the agent to be tested, dissolved in 30 ml of water, was administered through the Levin tube. The tube was clamped for 1 to 1½ hours and then sampling was resumed at 30 minute intervals for 3 hours (7 postmedication samples) For completion of the study, 42 subjects were given a placebo, 19 a single dose of 2.5 mg of reserpine, and 22 a single dose of 40 mg. of AP 396. The mean free acid, expressed as meq HCl per liter, as determined with Toepfer's reagent and 0.1 N NaOH (accuracy ± 5 meq/L), was calculated for each subject for the premedication (control) and for the postmedication period, as well as the difference between the mean free acidity of the control and the postmedication periods (the Δ mean free HCl). The mean free acidity value for each of the 3 medication groups was also calculated for the control and the postmedication periods (Table I)

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†Brand of crystalline alkaloid reserpine Rau-sed E R Squibb & Sons, New York, N Y.

‡Experimental antispasmodic and antigastric secretory drug (diethylaminoethyl beta-hydroxy x-phenylcyclohexane-acetate methobromide) , Abbott Laboratories, North Chicago, Ill

The second study was designed to measure the effects of various compounds upon the level of the blood sugar. The venous blood glucose (modified Somogyi method) (accuracy ± 5 mg. per cent) was determined 2 hours after a standard oral glucose load (1.75 Gm. per kilogram of ideal body weight). On various test days the oral glucose load was preceded 8½ and 2 hours before by the ingestion of 50 mg. cortisone, 10 mg. prednisone, and 10 mg. prednisolone by 21 subjects; on 1 other occasion it was preceded by 2 mg. 9-alpha-fluorohydrocortisone by 16 of the same subjects; and on still another occasion by 2.0 Gm. carbutamide by 12 of the same subjects. Each of the 21 subjects also had a control test (no medication prior to the ingestion of glucose).

TABLE I

SUBJECT NO.	FREE HCl MEQ./L.		Δ MEAN FREE HCl (MEAN POST-MED. MINUS MEAN CONTROL)	SUBJECT NO.	FREE HCl MEQ./L.		Δ MEAN FREE HCl (MEAN POST-MED. MINUS MEAN CONTROL)
	MEAN CONTROL (3 OBS.)	MEAN POSTMED. (7 OBS.)			MEAN CONTROL (3 OBS)	MEAN POSTMED. (7 OBS.)	
Placebo 1	40	36	− 4	Reserpine 1	15	60	+45
2	52	56	+ 4	2	17	55	+38
3	55	58	+ 3	3	13	39	+26
4	73	70	− 3	4	15	45	+30
5	34	42	+ 8	5	11	79	+68
6	17	11	− 6	6	42	72	+30
7	48	42	− 6	7	42	76	+34
8	51	57	+ 6	8	50	103	+53
9	51	39	−12	9	0	33	+33
10	0	23	+23	10	50	69	+19
11	60	66	+ 6	11	25	67	+42
12	8	11	+ 3	12	35	91	+56
13	2	16	+14	13	52	54	+ 2
14	0	1	+ 1	14	27	39	+12
15	5	0	− 5	15	68	92	+24
16	10	39	+29	16	18	70	+52
17	25	26	+ 1	17	18	60	+42
18	42	42	0	18	50	41	− 9
19	42	53	+11	19	0	14	+14
20	7	28	+21	Reserpine group mean	28.8	60.7	+32.0
21	12	11	− 1				
22	10	34	+24				
23	45	49	+ 4	AP 396 1	52	50	− 2
24	45	51	+ 6	2	40	37	− 3
25	15	46	+31	3	65	3	−62
26	77	36	−41	4	65	58	− 7
27	28	51	+23	5	23	3	−20
28	18	36	+18	6	33	14	−19
29	75	76	+ 1	7	38	24	−14
30	13	32	+19	8	30	21	− 9
31	27	63	+36	9	22	0	−22
32	53	46	− 7	10	23	3	−20
33	23	51	+28	11	27	0	−27
34	45	51	+ 6	12	18	3	−15
35	63	79	+16	13	38	23	−15
36	27	9	−18	14	33	14	−19
37	53	56	+ 3	15	30	3	−27
38	28	48	+20	16	13	3	−10
39	10	21	+11	17	22	6	−16
40	40	29	−11	18	20	13	− 7
41	22	54	+32	19	15	16	+ 1
42	35	69	+34	20	33	5	−28
				21	38	1	−37
				22	28	22	− 6
Placebo group mean	*33.0*	*41.0*	*+7.8 (Placebo control)*	AP 396 group mean	32.3	14.5	−17.5

Note: The difference between 41−33 = 8.0 and 7.8, 60.7−28.8 = 31.9 and 32.0, and 14.5−32.3 = −17.8 and −17.5 are due to "rounding off" of figures in calculations.

The third study included observations on 300 subjects There were 10 different eye drops tested For each medication there were 10 white subjects tested with blue eyes, 10 white subjects with brown eyes, and 10 Negro subjects with dark eyes The nature of the drops were unknown to both the investigators and the subjects The medications evaluated were normal saline (Placebo 1), 4 per cent boric acid (Placebo 2), 10 per cent Neosyneph rine, 3 per cent ephedrine, 2 per cent pilocarpine, 0 2 per cent scopolamine, 1 per cent Cyclogyl, 1 per cent atropine, 4 per cent homatropine, and 1 per cent Paredrine The pupil size was measured with a millimeter ruler at a standard distance from the eye, 3 drops of the medication to be tested were instilled in the eye, and the pupil size was again measured 40 minutes later, using the same technique Identical measurements were also carried out on the other eye, which received no drops The results of the 60 tests performed on the other eyes of those subjects receiving placebo drops were later utilized as the control observations In all, 303 tests were eventually completed in this study, an additional 3 tests being performed in Negroes with dark eyes using 2 per cent pilocarpine

More detailed information concerning the design and individual observations from the second and third studies are presented in separate reports[10] [11]

The sequential graphs presented here were all derived from the same basic formulas, the application of which has been variously described[1] [8] and recently further modified [9]

The calculations used for construction of these sequential graphs were identical with those modifications described for normally distributed measured variables[9] except here the Slope (S) was not subtracted from each value, thus allowing the lines of the graph to remain slanting A sequential graph so constructed will permit measurement of a significant change in one direction, as a significant increase over and above the established control values However, the plot lines of a drug inducing a significant decrease and of a drug inducing no significant change from the control observations will both cross the lower line of the graph and thus be rejected, but will not be differentiated one from the other This is inherent in the one sided alternative plan In order to further modify the graph to make this latter differentiation, one may expand the analysis as follows

The value $m_1 - m_0$ is known from the first calculation, as is the value of m_0 (9) One may then solve the following equation for m_2 (where m is the mean required in the treatment group for indicating a significant decrease in the values of the measured variables from those observed in the control group with a probability of $P = 0\ 01$ when $n_2 = 6$ $m_0 - m_2 = m_1 - m_0$

Allow α and β to remain the same, a and b will thus remain the same and h_1 and h_0 will remain the same

Note As an alternative method, the value of m_2 may be arbitrarily assigned, based upon the past experience of the investigator and his special knowledge in the field of study The average number of observations may then be calculated from the formula previously given[9] substituting $\bar{n}$ m for $\bar{n}$ m_1 and $m_0 - m_2$ for $m_1 - m_0$ The problem of increasing the average number of observations needed for a decision to an undesirably large size exists if one assigns the value for m_2 too close to that of m_0 We thus prefer the method given above for general use (allowing n_2 [n m_2] to arbitrarily be 6), as it usually leads to satisfactory clinical as well as statistical significance and generally permits the average number of observations to remain small

Then solve for a new slope

$S = \frac{m_0 + m_2}{2}$ (where S′ is the slope of the lower portion of graph)
Lower line $= -h_0 + S' n$
Upper line $= h_1 + S' n$

Solve again for $n = 0$, $n = 5$, $n = 10$, to obtain 3 plot points for construction of upper and lower lines of lower portion of graph

Note The upper line of the lower portion of the graph will now intersect the lower line of the upper portion of the graph The lines are arbitrarily not drawn to the left of the area of intersection, although they may be so drawn if desired

The resulting graph will now outline the zone of indecision and delineate "above," an area indicating a significant increase over the control values; "in the middle," an area indicating no significant change from the control values; and "below," an area indicating a significant decrease from the control values.

The abscissa of the graph constructed represents n_1 (number of observations of the test group) and the ordinate represents the ΣX (the sum of the measured variables in the test group). The individual measured variable of each test observation is plotted in summation fashion, one after the other for each test group as they are sequentially and randomly obtained, until a decision is reached. A decision is reached when the plot line of a test group crosses out from the zone of indecision into one of the three areas of decision.

The data utilized as the control in each of the three studies herein described were normally distributed.

RESULTS

In the study of gastric acidity, none of the mean "control" (premedication) values for free acidity (33.0, 28.8, 32.3) differed significantly from each other in any of the 3 medication groups (Table I). In view of the fact that the observations obtained after medication provided a two-sided alternative (a significant increase or decrease in gastric acidity as compared to the control) they appeared suitable for the attempt at interpretation by sequential analysis, modified as described above. The placebo postmedication period demonstrated a slight increase in mean free HCl of borderline significance when compared to its premedication control by *t*-test (P less than 0.1 and greater than 0.05) (Fig. 2). The placebo Δ mean free HCl values were thus used as the control data upon which to base the sequential analysis, since a significant difference from these values was the object of clinical search. Table II summarizes the basic data utilized for construction of all the sequential graphs presented in this paper.

TABLE II

BASIC DATA UTILIZED IN CONSTRUCTION OF THE SEQUENTIAL GRAPHS		GASTRIC FREE HCl STUDY	BLOOD GLUCOSE STUDY	EYEDROP STUDY
Control group	=	Data from placebo observations (Δ mean free HCl)	Data from no premedication tests	Data from observations on the other eye of those subjects receiving placebo drops
n_0 (number of control observations)	=	42	21	60
m_0 (control mean)	=	+ 7.8	78.29	+0.08
Sigma (Standard Deviation)	=	15.7	14.03	0.91
m_1	=	+26.96	+95.41	+1.19
m_2	=	−11.36	+61.17	−1.03
α	=	0.01	0.01	0.01
β	=	0.01	0.01	0.01
Intercepts:				
h_0	=	59.18	52.89	3.44
h_1	=	59.18	52.89	3.44
Slope (upper portion)	=	+17.38	+86.85	+0.63
Slope' (lower portion)	=	− 1.78	+68.73	−0.48

When the individual Δ mean free acidity values (mean postmedication minus mean control) are plotted from Table I for each subject in sequential fashion for each drug treatment group as illustrated in Fig. 1, reserpine is accepted as a significant stimulator agent on the first 4 observations, and AP 396 is accepted as a significant inhibitor agent on the first 7 observations.

The experiment was continued to gain larger numbers of observations (83 total) in the various medication groups and these results were analyzed

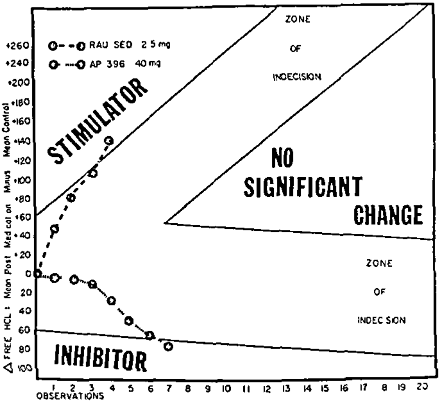

Fig. 1—The sequential graph illustrating that reserpine was found to be a significant stimulator of gastric acidity on 4 observations and AP 396 a significant inhibitor of gastric acidity on 7 observations utilizing the Δ mean free HCl observations from the placebo group as the control The abscissa represents n (the number of observations) and the ordinate the Σ X (the sum of the Δ mean free HCl of each observation) These are plotted in sequential and summation fashion for each subject in each drug test group until an answer is obtained

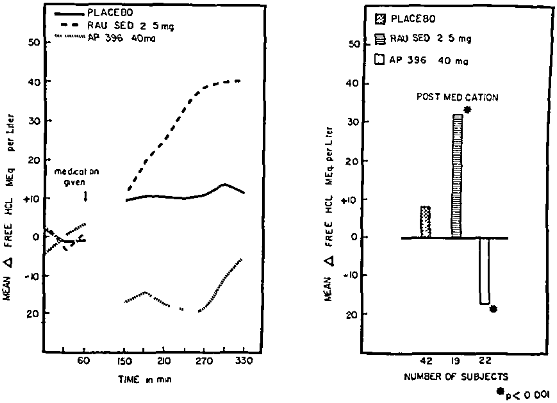

Fig 2—*Left*, The mean Δ free acidity, after all 83 tests were completed, (mean of postmedication observations minus mean control) for each time interval for each of the 3 drug treatment groups in the control (premedication) and postmedication period. *Right*, The mean Δ free acidity after all tests were completed (mean of postmedication observations minus mean of control observations) for each of the 3 drug treatment groups The P values are from *t*-tests using the mean Δ free HCl of the placebo group as the control

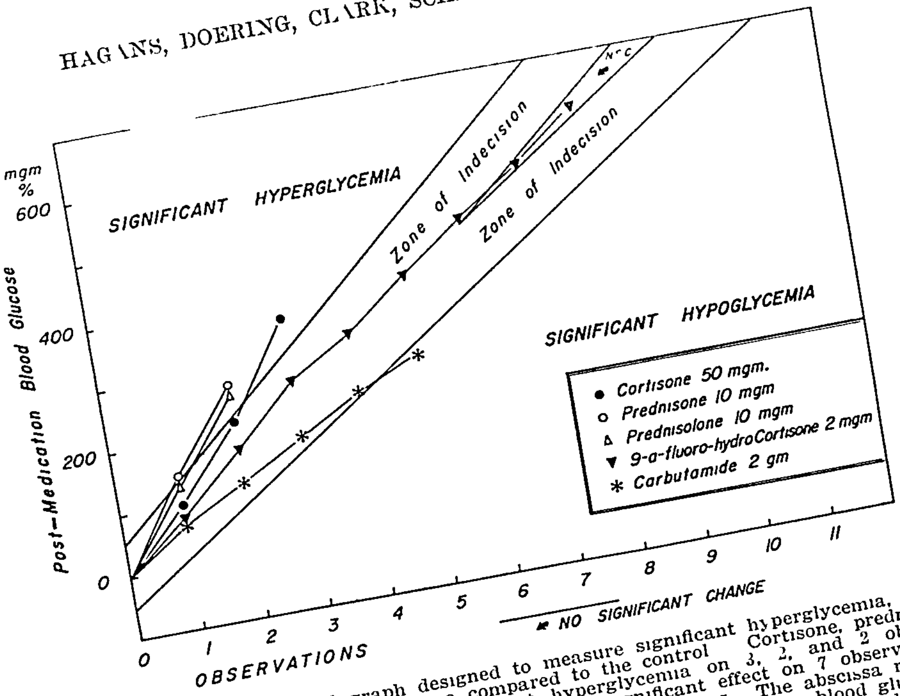

Fig. 3.—The sequential graph designed to measure significant hyperglycemia, no significant change or significant hypoglycemia compared to the control. Cortisone, prednisone and prednisolone were found to induce significant hyperglycemia on 3, 2, and 2 observations respectively, 9-alpha-fluorohydrocortisone to have no significant effect on 7 observations and carbutamide to induce significant hypoglycemia on 5 observations. The abscissa represents n (the number of observations) and the ordinate the ΣX (the sum of the blood glucose values observed in the drug test groups). The results are plotted in sequential and summation fashion for each drug test group until an answer is obtained.

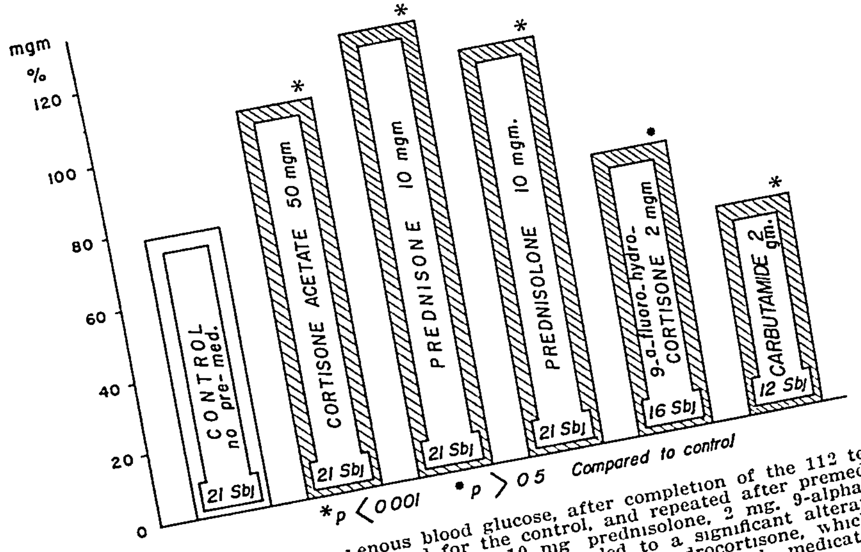

Fig. 4.—The group mean venous blood glucose, after completion of the 112 tests, 2 hours after ingestion of an oral glucose load for the control, and repeated after premedication with 50 mg. cortisone acetate, 10 mg. prednisone, 10 mg. prednisolone, 2 mg. 9-alpha-fluorohydrocortisone, and 2.0 Gm. carbutamide. All medications led to a significant alteration in blood glucose levels compared to the control except 9-alpha-fluorohydrocortisone, which induced no significant change. The P values are from *t*-tests comparing each medication group to the control.

by a standard statistical technique (*t* test) The rise in the level of the mean free HCl observed in the reserpine postmedication period (+32 0 meq /L) was significantly different from the placebo control (+7 8 meq /L) (P less than 0 001) and the fall observed in the AP 396 postmedication period (–17 5 meq /L) was also significant when compared to the placebo control (P less than 0 001) (Fig 2)

For the venous blood glucose studies, the sequential graph was constructed from the absolute values of the control data (no premedication) according to the technique previously described under Methods The analysis utilizing this graph is presented in Fig 3 The individual value for each subject's 2 hour blood glucose determination for each drug treatment group was plotted in sequential and summation fashion until an answer was obtained When the answer was evident after plotting the first test a second test was plotted to insure that the decision remained unchanged This technique permitted the conclusion that, compared to the control, cortisone induced a significant rise in blood glucose (only 3 tests required for decision), as did prednisone and prednisolone (2 tests with each required for decision), and that 9 alpha fluorohydrocortisone induced no significant change in blood glucose from the control observations (7 tests required for decision) The conclusion that carbutamide led to a significant fall in blood glucose was reached in 5 tests

The experiments were continued until there were larger numbers in the various medication groups (112 tests total), and the results were then analyzed by the *t* test Fig 4 summarizes the results of these further studies The P values for the *t* tests performed permit the conclusion that cortisone, prednisone, and prednisolone in the dosages employed, significantly elevate the blood glucose 2 hours after a standard oral glucose load compared to the control (P less than 0 001 in each instance) and that 2 mg of 9 alpha fluorohydrocortisone induces no significant change in the blood glucose value compared to the control (P greater than 0 5) Carbutamide in a dose of 2 0 Gm leads to a significant fall in the blood glucose level (P less than 0 001)

The sequential graph for analysis of the eyedrop tests, also constructed according to the technique described above, was based on the variation observed in the 60 control observations carried out in the other eyes of those subjects receiving placebo drops Fig 5 (upper half) illustrates that the conclusion that scopolamine, atropine, Paredrine, homatropine, and Cyclogyl induced significant mydriasis in white blue eyes white brown eyes, and Negro dark eyes was reached in each instance with only 2 to 5 individual tests plotted in sequential and summation fashion for each drug group Fig 5 (lower half) illustrates that the conclusion that neither placebo induced a significant change in pupil size compared to the control observations in white blue eyes white brown eyes, or Negro dark eyes was reached with only 7 to 10 tests plotted in similar fashion for each group tested Further the conclusion that Neosynephrine did induce significant mydriasis in all 3 eye type groups is reached in 4, 2, and 5 tests, respectively Of particular interest here is the conclusion that ephedrine induces significant mydriasis in white blue eyes was reached in 3 tests, and in white brown eyes in 7 tests, and the conclusion that it does not induce significant mydriasis in Negro dark eyes was reached in 9 tests. The conclusion that pilocarpine induces significant myosis in white blue eyes was reached in 4 tests, in white brown eyes in 4 tests, but in Negro dark eyes 11 tests were required for a decision.

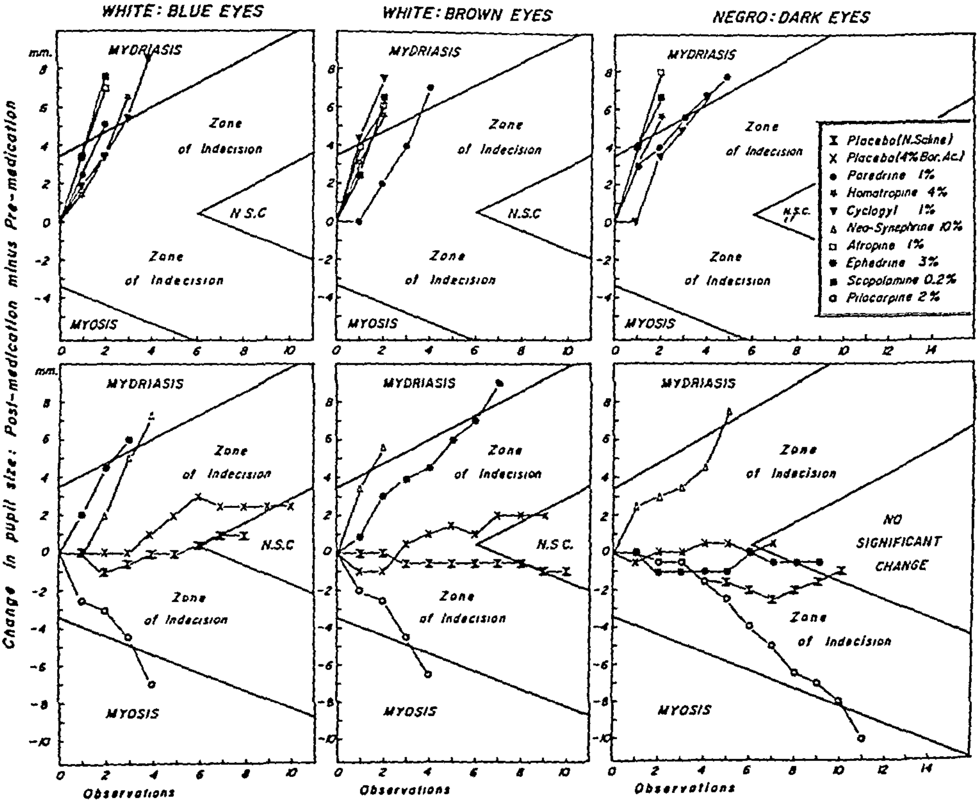

Fig. 5.—*Upper half,* The sequential graph constructed in order to measure significant mydriasis, no significant change or significant myosis compared to the control observations. Cyclogyl, Paredrine, scopolamine, atropine, and homatropine are found to induce significant mydriasis in all eye types with no more than 5 tests with any drug in any eye-type group being required in order to reach this decision. *Lower half,* The same sequential graph reproduced and illustrating that the decision that neither placebo induced a change significantly different from the control in all eye-type groups was reached on from 7 to 10 tests on every occasion, that the decision that Neosynephrine does induce significant mydriasis in all eye-type groups was reached on 4, 2, and 5 tests, and that pilocarpine does induce significant myosis in all eye-type groups was reached on 4, 4, and 11 tests. Ephedrine was found to induce significant mydriasis in white blue eyes on 3 tests, in white brown eyes on 7 tests, and was found to be totally ineffective in Negro dark eyes on 9 tests. The abscissa represents n (the number of observations) and the ordinate the ΣX (the sum of the changes in pupil size observed in each group after medication). The results are plotted in sequential and summation fashion for each drug test group in each eye-type group until an answer is obtained.

The experiments were continued until larger numbers were gathered in the various medication groups (303 total) and the results were then analyzed by the *t*-test. Fig. 6 illustrates these results. The mean change in pupil size for each drug treatment group is plotted in the upper portion of the graph for white blue eyes, white brown eyes, and Negro dark eyes, as well as for the control observations individually by eye type and also combined, and for the placebo observations individually by eye type. No significant differences between the results of the placebo tests and those of the control observations were demonsrated (P less than 0.5 and greater than 0.1). Scopolamine, atropine, and homatropine, as well as Neosynephrine, Paredrine, and Cyclogyl, induced significant dilatation of the pupil in all 3 eye-type groups (P less than

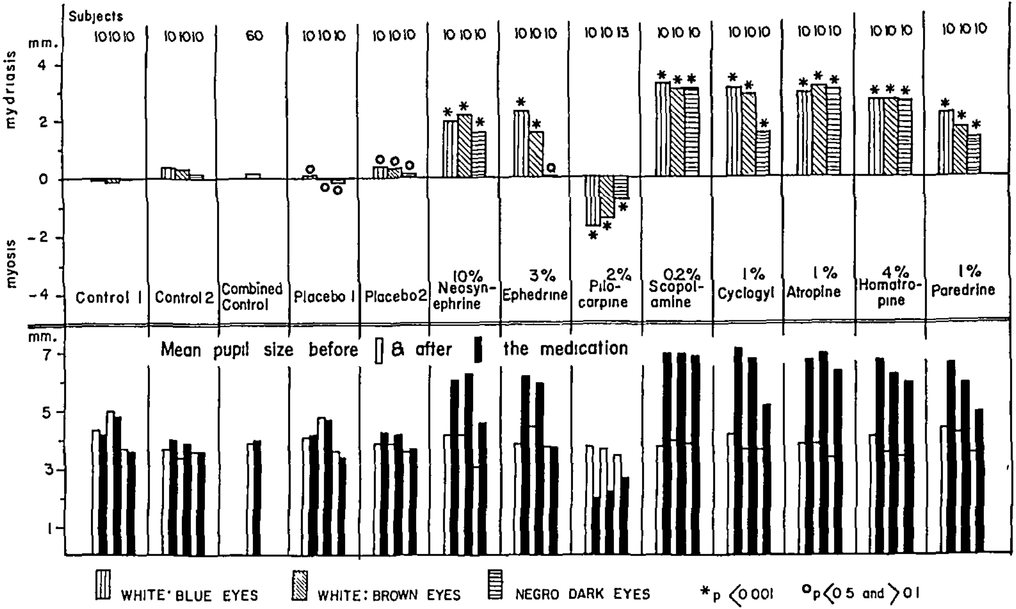

Fig 6.—The mean change in mean pupil size (in millimeters) following medication after 303 tests were completed; for the control groups, changes are expressed individually by eye type and also for the results of all 60 control tests combined, for the placebo trials expressed individually by eye type, and for each test agent expressed individually by eye type None of the results of the placebo tests differed significantly from the control observations Neosynephrine, Cyclogyl, Paredrine, scopolamine, atropine, and homatropine induced significant mydriasis in all eye types, although the first 3 were slightly less potent in Negroes Pilocarpine induced significant myosis in all eye types but was considerably less potent in Negroes Ephedrine induced significant mydriasis in white subjects with blue eyes and brown eyes, but failed completely in Negro subjects with dark eyes The P values are from t-tests using the 60 observations on the untreated eyes of the placebo groups as the control The lower portion of the graph indicates the actual mean pupil size in millimeters for each eye-type group in each medication group before and after medication.

0.001 in each instance), though Cyclogyl and, to a lesser extent, Neosynephrine and Paredrine were somewhat less effective in the Negro dark eyes. Ephedrine induced significant mydriasis in white blue eyes and brown eyes (P less than 0.001 in both instances) but failed to significantly alter pupil size in Negro dark eyes (P less than 0.5 and greater than 0.1). Pilocarpine induced significant myosis in all 3 eye-type groups (P less than 0.001 in each instance) but was considerably less potent in Negro dark eyes (it required 11 observations to reach a decision here on the sequential graph).

SUMMARY AND CONCLUSIONS

Three studies involving the measurement of the pharmacodynamic effects of various agents on human subjects are presented. A method is described adapting the technique of sequential analysis to the interpretation of the data obtained in these studies, all of which included the possibility of a significant increase and a significant decrease after treatment from the measured variables in the control group. The data from the control group in each study were normally distributed, a criterion which must be met before sequential analysis as herein described can be utilized.

Eighteen different pharmacodynamic agents acting on 3 widely differing bodily processes were tested in 498 separate trials appropriately controlled by a placebo double-blind technique. In each instance sufficient observations were made to allow further analysis of the data by a standard statistical technique. The sequential graphs, however, yielded valid inferences after a relatively small number of observations, which were in each instance consistent with the inferences later drawn from the more commonly used and reliable *t*-test.

The sequential plan described should be applicable in many areas of clinical investigation where measured variables are involved and a bidirectional or two-sided alternative exists. Sequential analysis should become an important and useful tool in therapeutic research.

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THE EFFECT OF GRADIENT TYPE HYDROGEN ION CONCENTRATION PLATES ON ANTIBIOTIC SENSITIVITY TESTS

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The method of determining bacterial sensitivity to antibiotics by the use of dried, impregnated filter paper disks on solid media is standard procedure in many clinical laboratories However, it is well recognized that a number of variable factors may influence the results of this type of test These include (1) variations in the nature and composition of the medium, (2) the depth of the agar, (3) the hydrogen ion concentration of the medium, (4) the size and age of the bacterial inoculum, (5) the duration of incubation, (6) the amount of surface moisture on the agar, and (7) the concentration of antibiotic in the filter paper disk Each of these factors independently or in combination may affect the final result and close attention to technique is required if accurate and reproducible results are to be obtained

Of these variable factors the influence of changes in the pH of the medium is of considerable importance The present study was undertaken in view of the observation of conflicting results obtained with different batches of agar These were found to be due to differences in the final pH of the media The method employed consists of a modification of the gradient type agar plate described by Szybalski[1] The plates were prepared according to the method of Sacks[2], the gradient pH was produced by the incorporation of different buffer solutions

MATERIALS AND METHODS

Antibiotics—Stock solutions of crystalline penicillin G (1,000 units per milliliter), chlortetracycline, oxytetracycline, tetracycline, streptomycin sulphate and chloramphenicol (each 1,000 μg per milliliter) were prepared in sterile distilled water Solutions of erythromycin and spiramycin were prepared by dissolving 50 mg of the pure substance in 1 0 ml of ethanol and then diluting with sterile distilled water to a final concentration of 1,000 μg per milliliter

The stock solutions were stored in the Deepfreeze and suitable dilutions were prepared in sterile distilled water as required The potency of the stock solutions remained constant during the period of the investigation

Gradient pH Plates—Sterile molar solutions of K_2HPO_4 and $KH\,PO_4$ were prepared The former was added to 10 ml of melted and cooled nutrient agar (1 10 v/v) which was then poured *into a sterile Petri plate* The agar was allowed to *solidify with the plate held* in an inclined position so that a wedge shaped area of agar was produced This was most conveniently done by resting the bottom of the plate near its center, on a piece of glass tubing A second aliquot of melted agar containing $KH\,PO_4$ (1 10 v/v) was then poured on top of the first layer with the plate held in a horizontal position Diffusion of the buffer solutions took place rapidly between the two layers and the plates were ready for use in 2 to 3

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hours. The range of pH across the plates was determined by laying strips of filter paper containing indicator solutions, across the surface. The method gives a satisfactory and uniform change of pH from approximately 5.4 on one side to 8.0 on the other.

All plates were used on the day of preparation although it was found that storage for 36 hours at 4.0° C. did not affect the results.

Filter Paper Strips.—Sterile strips of filter paper (Whatman No. 42), 87 mm. by 6 mm., were cut and soaked in the appropriate antibiotic solution. The excess fluid was allowed to drain off and the strips were then placed across the central portion of the gradient plates, following inoculation of the plates with the test organism.

Inoculum.—The bacterial inoculum consisted of 6 drops of a 6-hour digest broth culture added to 3 ml. melted and cooled 1.0 per cent nutrient agar. The agar was then poured across the surface of the plate and allowed to solidify. Most of the tests were carried out with *Staphylococcus aureus* (Oxford strain). A number of other organisms were also tested and gave identical results with the antibiotics to which they were sensitive (*Salmonella typhi, Escherichia coli,* etc.).

All plates were incubated overnight at 37.0° C. and the results noted. With each organism a control gradient plate was included, omitting the antibiotic, to make certain that growth was equal and satisfactory over the pH range.

RESULTS

Figs. 1 to 10 show the patterns of inhibition produced with the various antibiotics and with varying concentrations of the same antibiotics.

1. *Comparison of Different Antibiotics.*—It will be seen that the various antibiotics fall into three main groups according to the nature of the zone of inhibition.

Group 1 (Figs. 1 and 2) consists of penicillin and chloramphenicol. Here the degree of diffusion is more or less equal over the whole pH range so that the growing edge of the inoculum is parallel to the antibiotic strip. At pH levels below that of the gradient plates penicillin was found to have a more marked effect since it is a fairly strong acid. In the case of chloramphenicol (Fig. 2) there is an appearance of a larger zone of inhibition at both edges of the plate. This appearance was noted with other antibiotics and is probably the result of two factors; (1) the buffering effect is poor at the edge of the plate and (2) the thickness of the agar is less than in the rest of the plate due to the curvature of the bottom. This latter point was investigated further by preparing agar plates with the agar sloped across the plate in a wedge shape. Antibiotics strips were then placed on the surface as described above and the plates inoculated. Diffusion was found to be considerably more marked in the thin section of the agar.

Group 2 (Figs. 3 to 9) consists of those antibiotics whose effect is most marked on the alkaline side, i.e., streptomycin, erythromycin, and spiramycin. Group 3 is represented by chlortetracycline (Fig. 10) where the maximal effect is on the acid side. Tetracycline and oxytetracycline were found to give similar patterns of inhibition to chlortetracycline.

2. *Effect of Varying Concentrations of Antibiotic.*—A comparison of Figs. 3 and 4; 5, 6, and 7; and 8 and 9 shows the effect of varying the concentration of antibiotic. With those drugs whose effect is most marked on the alkaline side, the effect of decreasing concentrations is to increase the pH level at which

the end point of no inhibition of growth is apparent With increasing concentration there appears to be a direct relationship between the percentage inactivation of antibiotic and its concentration, at any particular pH level

Fig 1

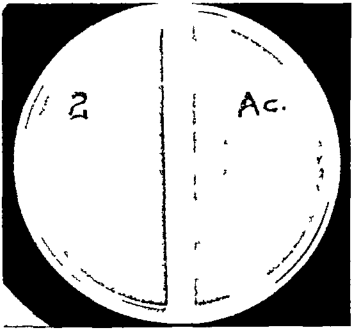
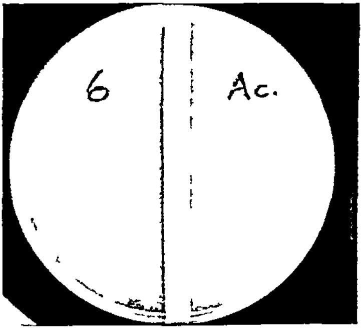


Fig 2



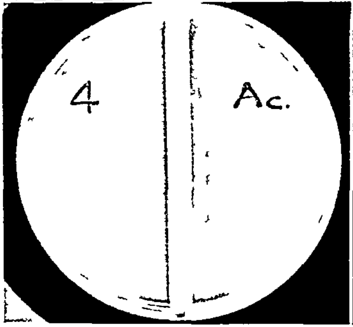


Fig 3

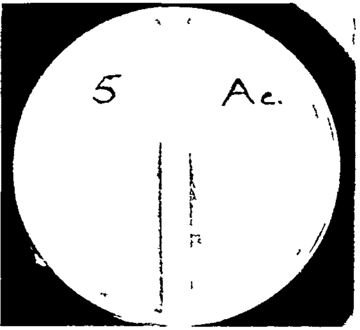


Fig 4

Fig 1—Effect of penicillin (100 units per milliliter) incorporated in strip Ac = acid side of plate There is practically no difference in the zone of inhibition in the acid and alkaline regions Test organism *Staph aureus* var *Oxford*

Fig 2—Chloramphenicol at a concentration of 1 000 µg/ml Here the zone of inhibition is considerably narrower than in Fig 1 but otherwise has similar features Test organism *Staph aureus* var *Oxford*

Figs 3 and 4—The antibiotic strip in Fig 3 contains 5 000 µg/ml of streptomycin sulphate and 1 000 µg/ml in Fig 4 Fig 4 shows complete resistance of the test organism (*Staph aureus* var *Oxford*) in the acid region

However, changes in the concentration do not appear to affect the size of the angle of the zone of inhibition from the strip This is particularly well shown in Figs 5 and 6 where a tenfold increase in concentration has merely altered the pH level at which no inhibition of growth becomes apparent strips where they had been in contact with the alkaline side of the agar, as in the case of the tetracycline drugs. The effect of an unsatisfactory hydrogen-ion concentration was thus to inactivate the antibiotic and not merely to hinder its diffusion.

DISCUSSION

The very wide differences which exist in the optimal hydrogen-ion concentration for activity in the case of the different antibiotics, indicates clearly the importance of strict control of the pH of the medium employed.

False impressions may otherwise be obtained regarding the likely effect of an antibiotic in vivo. This is particularly the case where the concentrations employed in sensitivity tests in vitro bear little relation to the concentrations which are attainable in the blood and tissue fluids.

Among the basic antibiotics other than streptomycin, both streptothricin and proactinomycin are also more active with increase of pH. In the case of the latter for example Geiger and associates[3] have shown that an increase of pH from 6.5 to 7.5 has an effect equivalent to a fourfold increase in the concentration of the antibiotic. With regard to penicillin, Stokes[4] states that the zones of inhibition do not vary between pH 6.0 and 8.0. This agrees with our observations (Fig. 1). Foster and Woodruff,[5] however, found that a shift of pH from 7.0 to 5.5 resulted in an apparent threefold increase in effect in the case of penicillin.

The choice of an antibiotic in the control of a focus of infection depends on factors involving host-parasite and host-drug relationships in addition to the demonstration of sensitivity of the causal organism. Changes in hydrogen-ion concentration in various parts of the body, for example, may be great enough to influence the activity of the antibiotic. In the case of a urinary infection it is obviously essential to render the urine alkaline if streptomycin is to be given. Such a procedure would be contraindicated, however, if the tetracycline drugs were to be employed. Similarly, where there is a focus of necrotic tissue. produced as the result of infection, the pH of the region tends to be highly acid. In such a situation a drug of the tetracycline group may prove to be more effective than one whose optimal activity lies on the alkaline side. In the case of systemic infections where the effect of the antibiotic is dependent on its activity in the blood and tissue fluids at pH of approximately 7.4, it would seem that relatively greater amounts of the tetracycline drugs would be required than in the case of streptomycin, erythromycin or spiramycin. The relative ineffectiveness of the tetracycline drugs in typhoid fever as compared with chloramphenicol may partly be explained by the fact that the pH of the tissue fluids and blood will tend to decrease the activity of the former. In vitro estimations show that the minimum inhibitory concentrations of these antibiotics for *S. typhi* are very similar, indicating that host factors are of great importance in determining the effect of antibiotics in vivo.

The principle of the gradient-type plate has been applied to other types of investigation. Sacks[2] made use of it to study the effect of varying pH levels on the growth of organisms Streitfeld and Saslaw[6] have described a modification for determining the sensitivity of organisms to paired combinations of antibiotics

SUMMARY

1 The effect of antibiotics, at varying hydrogen ion concentrations on *Staphylococcus aureus* (var *Oxford*) is described The method employed consisted of a gradient type pH plate

2 The results indicate the importance of adequate control of pH in media which is to be used for determining sensitivity of organisms to antibiotics

We are indebted to May & Baker (S A) for a supply of pure spiramycin and to Parke, Davis & Co , for the pure chloramphenicol

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LABORATORY METHODS

NEUTRON ACTIVATION ANALYSIS OF SODIUM IN BLOOD SERUM

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NEUTRON activation analysis offers an essentially nondestructive means of quantitatively estimating certain anions and cations. As part of an investigative program on the applications of neutron activation analysis to biology and medicine,[1] this laboratory has studied the determination of sodium in blood. The procedure has been found to be rapid and reproducible, and has the added advantage of entailing minimal handling of the specimen. Since the material is not consumed, it may be reassayed or used for other studies.

The steps involved in the operation consist of: irradiation of a sample in a low intensity neutron flux, measurement of the induced radioactivity, and comparison of the radioactivity with that of a standard handled in identical fashion. This report describes the method and the results obtained after "contaminating" short half-life isotopes have decayed.

PRINCIPLE

When tissue is exposed to a neutron flux, interactions occur between bombarding particles and target nuclei. With fast neutrons, multiple nuclear events, such as (n,γ), (n,p), and (n, 2n), can occur. However, when thermal neutrons are employed, they almost exclusively engender (n,γ) interactions, and other processes may be neglected. Following thermal neutron bombardment, radioactivity induced in any particular element within the specimen is given by the expression[1]:

$$M = \frac{(1.63 \times 10^{-5})\ n\ .\ \alpha A\ .\ W\ .\ f\ .\ (1 - e^{-\lambda} t_1)\ (e^{-\lambda} t_2)}{A_t}. \qquad 1.$$

M is the activity in microcuries; n the flux in neutrons per square centimeter per second; α A the isotopic neutron capture cross section in barns; W the weight of the element in grams; f the fractional abundance of the precursor isotope in the element; A_t the atomic weight of the element; λ the disintegration constant of the product radioactive isotope; t_1 the time of sample exposure to the neutron flux; and t_2 the interval elapsed from the cessation of irradiation to the beginning of analysis.

Equation 1 can be solved for the neutron flux ($n = M.A_t/$ etc.). Hence if a material of known composition and an unknown are exposed to the same flux, they may be

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equated. If both samples were subjected to an identical flux for a similar time interval, and then analyzed simultaneously, they would be related by the expression

$$\frac{Ms}{Ws} = \frac{Mu}{Wu} \qquad 2$$

where M is the radioactivity, W is the weight of the element, s represents the standard, and u represents the unknown. In other words, the radioactivity induced under such conditions would be proportional to the quantity of material originally present.

When only one device for detecting radioactivity is present, the standard is analyzed first, and the unknown is usually counted subsequently. In this situation, the more general one, activity in the standard must be corrected to the time the unknown is counted.

$$\frac{Ms\ (e\ \lambda\ t_0)}{Ws} = \frac{Mu}{Wu} \qquad 3$$

The time elapsed between the onset of analysis of the standard, and the beginning of the same procedure on the unknown, is represented by t_0.

Equation 3 is the mathematical expression used in the present analysis. The measurement of the radioactivity present may be performed by any suitable device and expressed in any units, as long as the same method is utilized for the standard and the unknown. Two techniques for determining radioactivity were employed: counting in a well type scintillation counter, and utilizing the area under the gamma ray spectral curve.

METHODOLOGY

Samples consisted of dried human serum of known composition.* Polyethylene tubes, whose outer dimensions measured 15 × 35 mm, were washed in nitric acid and distilled water, then air dried. Each sample was placed in one of these containers, and heat sealed. Tubes were again washed, to remove contaminants resulting from handling.

Loaded tubes were placed in a polyethylene bag and lowered into the glory hole of a pool type nuclear reactor.† Flux densities varied from 2 × 10 neutrons/cm^2/sec, to 2 × 10^9. Reasons for this choice of values (which include their availability from small nuclear reactors, Van de Graaff—beryllium sources and radium—beryllium systems) have been presented elsewhere.[1] Time of irradiation was 20 to 60 minutes, depending upon the flux.

The actual value of the neutron flux can be calculated by inserting a known weight of sodium or other foil into the reactor, measuring the induced radioactivity after a known period of time, and substituting in equation 1. Practically however, this step is of no consequence, since standard and sample are both subjected to the same flux.

After irradiation, samples were counted in a well type scintillation counter, and then further analyzed in a Nuclear Model 1810 pulse height analyzer and well type crystal. The base line voltage of the analyzer was continuously varied by a motorized potentiometer. Pulses within 1.0 volts above the base line were fed through a scaler and rate meter to a rectilinear recording galvanometer. The potentiometer was driven at a rate of 1 turn/50 minutes. The time axis was also the axis of base line voltage, and a distance of 10 inches on the recording paper corresponded to scanning from 0 MEV to 4 MEV.

The area under the gamma ray curve, rather than peak height measurement, was utilized for estimating the quantity of radioactivity. This was done for two reasons. First the height of a particular peak might partially be due to the photo emission of a contaminant. When averaged over a larger total area such a contribution becomes negligible. Second, when using a narrow discrimination (a window only one volt wide) the peak height may not be perfectly linear, this must be checked for each system used.

"Noise" on the gamma ray spectrometer is low. As long as the 1 K or a higher scale is used on the count rate meter, no correction is necessary for background recorded by the gamma ray spectral curve. When the 300 scale is employed, a background correction is necessary. Radioactivity induced in the polyethylene tubes was negligible, and possessed a short half-life (perhaps Cl^{38}). No counts from the container registered at the sodium photoelectric peak.

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*Versatol, Warner Chilcott Laboratory Supply Division, Morris Plains, N. J.

†We are indebted to the Staff of the Nuclear Reactor Project, Naval Research Laboratory, Anacostia, Md., for the service irradiations.

To calibrate the apparatus, Na^{24} of known activity (purchased from the A.E.C.) was utilized. The system was also calibrated for internal stability and reproducibility, by introducing a small concentration (about 0.1 microcurie) of Na^{24}, and obtaining repeated gamma ray spectral curves. The area under each 50-minute tracing should have been 0.963 times the area under the preceding curve (0.963 represents the decay of Na^{24} during 50 minutes). In Table I are listed the measured, as well as the theoretically calculated areas during a trial run. The error in calculating the successive areas by use of a planimeter (vide infra) was below 2 per cent.

TABLE I. AREA BENEATH GAMMA RAY SPECTRUM OF A SAMPLE OF NA^{24}, OBTAINED BY HAVING SPECTROMETER TRACE REPEATED 50-MINUTE CURVES

TIME IN MINUTES	AREA IN SQUARE INCHES THEORETICAL	MEASURED	DIFFERENCE IN SQUARE INCHES	DIFFERENCE IN PER CENT
0- 50	—	7.81	—	—
50-100	$7.81 \times (0.963)^1 = 7.52$	7.41	0.11	1.5
100-150	$7.81 \times (0.963)^2 = 7.24$	7.29	0.05	0.7
150-200	$7.81 \times (0.963)^3 = 6.97$	6.90	0.07	1.0
200-250	$7.81 \times (0.963)^4 = 6.71$	6.63	0.08	1.2
250-300	$7.81 \times (0.963)^5 = 6.46$	6.40	0.06	1.0

Theoretical calculations[1] have indicated that exposure of 33 mg. sodium (the approximate quantity in 10 ml. serum) to a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for a period of one hour, will result in the production of 0.14 μc of Na^{24}. To test the ability of the recording system to distinguish between different quantities of radiosodium, quantities of the isotope varying from 0.05 to 0.35 μc were employed.

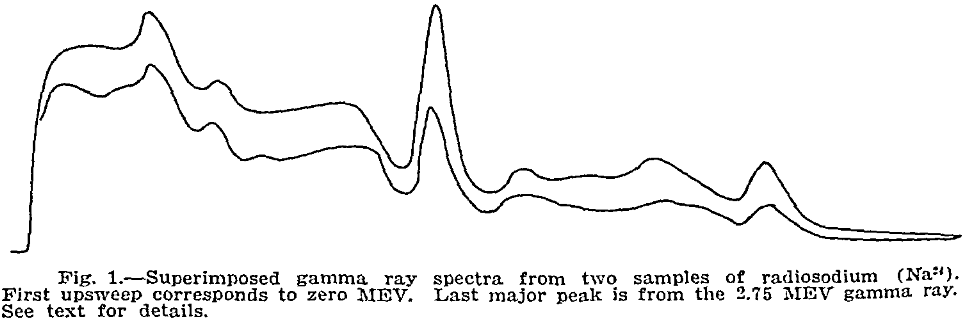

Fig. 1.—Superimposed gamma ray spectra from two samples of radiosodium (Na^{24}). First upsweep corresponds to zero MEV. Last major peak is from the 2.75 MEV gamma ray. See text for details.

A result of such a study is shown in Fig. 1. The lower curve represents the spectrum obtained utilizing 0.070 μc Na^{24}. Instrument settings were: analyzer: gain 1, 1 volt window; count rate meter: 10 second response, 10 K scale. By means of a Keuffel and Esser (4236) compensating polar planimeter, the area under the curve was determined. The "cutoff point" on the graphs has been considered to be the end of the photoelectric peak corresponding to the 2.75 MEV gamma ray. It must be recognized that decay was also occurring throughout the 50-minute scan, but this is neglected in subsequent calculations since the same decay was occurring during the tracing of later samples. That is:

$$\text{Amount in A (Decay during 50 minutes)} = \frac{\text{Y (Amount in B)}}{\text{(Decay during 50 minutes)}}. \qquad 4.$$

For the particular curve illustrated, the area was 3 89 square inches Fifty minutes later, due to radioactive decay, the area traced would have theoretically been

$$A = A_0\, e^{\lambda\, t}$$
$$A = (3\ 89)\ (e^{0\ 0462 \times 0\ 833})$$
$$A = 3\ 74 \text{ square inches}$$

The factor 0 0462 is the disintegration constant of Na^{24} expressed in reciprocal hours, and 0 833 is the decimal equivalent of 50/60 hours

The upper curve in Fig 1 (enclosing 7 35 square inches) is the spectrum from 0 14 μc Na^{24}, run 50 minutes after the first tracing Twice the corrected first area (representing 0 07 μc) should equal the area under the second curve (representing 0 14 μc) Thus it does, within $\frac{7\ 35}{2 \times 3\ 74} \times 100$, or 98 2 per cent Errors in pipetting, recording radioactivity by the spectrometer, and determining the area with a planimeter, were less than 2 per cent On repeated trials, the error has not exceeded 3 per cent

When pure samples are employed, counting in a well type scintillation counter is more convenient and expedient than use of a gamma ray spectrometer However, if there is uncertainty as to the number of radioactive constituents in a sample, gamma ray spectral curves are essential Hence, in the initial experimental work, a spectrometer was employed This method is applicable to any gamma emitting isotope As will be shown, 12 hours after irradiation of blood serum, Na^{24} is the only major gamma ray emitting isotope remaining

RESULTS

After one hour bombardment of 10 ml serum of normal composition by a flux of $2\ 5 \times 10^{8}$ neutrons/cm ^{2}/sec, the following gamma ray emitting isotopes are present[1] to the extent of 30 disintegrations per minute (d p m) or greater Na^{24}, Cl^{38}, Br^{80}, Br^{80m}, Br^{82}, I^{128}, K^{42}, Ca^{49}, Cu^{64}, Cu^{66}, Mg^{27}, and depending upon the initial concentration, perhaps Co^{60m} and Mn^{56} (m refers to the first of two metastable isomers) Thus, *the area under the gamma ray curve immediately* after neutron bombardment, contains contributions from a multitude of radio nuclides The general shape of the curve is that of pure Na^{24}, although its area is not determined by radiosodium alone Estimation of the quantity of sodium originally present in the specimen cannot be accurately performed by estimating the area bounded by the curve, since that quantity is a complex representation of many isotopes

TABLE II GAMMA EMITTING ISOTOPES REMAINING 12 HOURS AFTER BOMBARDMENT OF 10 ML SERUM* BY $2\ 5 \times 10^{8}$ NEUTRONS/CM ^{2}/SEC, FOR ONE HOUR*

ISOTOPE	HALF LIFE IN HOURS	GAMMA† FACTOR	D P M AT T 12‡	TOTAL GAMMA/MIN	RATIO $100 \times \frac{\text{GAMMA/MIN ISOTOPE}}{\text{GAMMA/MIN } Na^{24}}$
Na^{24}	15 0	2 00	184 640	369,280	100 00
Cl^{38}	0 62	0 47	2	1	0 00
Br^{80m}	4 6	2 00	388	776	0 21
Br^{82}	35 9	1 00§	215	215	0 06
K^{42}	12 5	0 20	983	196	0 05
Cu^{64}	12 8	0 38‖	116	44	0 01

$$\frac{Na^{24}\text{ gamma/min}}{\text{Total gamma/min}} = \frac{369\ 280}{370\ 512} \text{ or } 99\ 6 \text{ per cent}$$

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* Assumed composition of the serum sodium 33 mg chlorine 34 mg bromine 0 1 mg *potassium 1 6 mg copper 10 ^{2} mg*

†Average number of gamma rays per disintegration

‡Disintegrations per minute 12 hours after removal of the sample from neutron flux

§Assumed value may be in error

‖Due entirely to positron annihilation Br^{80} refers to the first of two metastable forms

Twelve hours after removal of the sample from the neutron flux, the "contaminating" nuclides have markedly decreased (Table II). From the Table, it can be observed that gamma rays from Na^{24} constitute nearly 99.6 per cent of the gamma emissions from the irradiated blood serum. The quantity of sodium originally present in the sample may then reasonably be calculated by determining the radioactivity at this time, and substituting into equation 3.

Any suitable detection device could have been used to count the 12-hour specimen. Use of a well-type scintillation counter gave good results. A gamma ray spectral curve was obtained on this specimen simply to rule out the possibility of a contaminant being present. As an example: twenty-one hours after the exposure of 10 ml. dried human serum (containing 32.5 mg. sodium) to a flux of 1.29×10^9 neutrons/cm.^{2}/sec. for thirty minutes, the gamma ray spectrum was obtained. Instrument characteristics were: analyzer: 1 volt window, gain 1; count rate meter: 2 second response, 3 K scale. Area under the curve was 5.88 square inches. An "unknown" that had been subjected to the same flux for an identical period, was then (50 minutes after the start of the first sample) placed in the gamma ray spectrometer. Area under this curve was 5.18 square inches. By use of Equation 3:

$$\frac{5.88\ (e^{-0.0462 \times 0.833})}{32.5\ \text{mg.}} = \frac{5.18}{\text{Wu}}$$

Wu = 29.7 mg. Actual weight of sodium in the unknown sample was 30.1 mg. (determined by the manufacturer, employing flame photometry). Agreement between the values obtained by activation analysis and by flame photometry was $\frac{29.7}{30.1} \times 100$ or 98.7 per cent.

Presented in Table III are six consecutive analyses of sodium in dried blood serum as obtained by activation, and the corresponding value by flame photometry. Difference between the two techniques was less than 3 per cent.

TABLE III. ESTIMATION OF SODIUM IN BLOOD SERUM BY NEUTRON ACTIVATION ANALYSIS, AS COMPARED WITH FLAME PHOTOMETRY. SAMPLES RUN 12 TO 21 HOURS AFTER COMPLETION OF IRRADIATION

SODIUM IN MG.		DIFFERENCE	
FLAME PHOTOMETRY	ACTIVATION ANALYSIS	IN MG.	IN PER CENT
16.3	16.5	0.2	1.2
20.8	20.3	0.5	2.4
25.9	26.3	0.4	1.5
30.1	29.7	0.4	1.3
30.4	30.1	0.3	1.0
32.5	32.9	0.4	1.2

DISCUSSION

Neutron activation analysis is an essentially nondestructive analytic procedure. Even if the neutron flux induced protein denaturation, there would be no interference with the analysis of radioanions and radiocations, as long as the entire specimen was counted. An infinitesimal number of nuclei, when compared with the total number in the sample, are made radioactive by neutron bombardment. Hence, the material may be assayed repeatedly, or used for other tests. A sodium analysis can be performed on blood serum, and the sample will still be intact for later studies. Studies have also been performed using liquid plasma and serum. Aside from the fact that they require more space in the nuclear reactor, they give results identical to those obtained from dried serum.

The predominance of the Na^{24} gamma emission may be taken advantage of in additional ways. Thus, by shielding the sample, to reduce the activity of other gamma rays, and counting at the 2.75 MEV photoelectric peak, it is possible to obtain an estimation of the quantity of radiosodium after a period of irradiation as short as ten minutes. Our experience with this is limited but has been promising. Reiffel and Stone[2] used a somewhat similar technique for estimating sodium in muscle. If sodium is but one of the electrolytes to be quantitated in serum, the irradiated sample may be fractionated by means of suitable anion and cation exchange resins. This technique has proved useful, and is being investigated further.

The gamma ray spectrometer—area technique, as discussed, is applicable to any gamma emitting isotope. The procedure is of great value when a radiocontaminant is suspected. First, the gamma ray peak characteristic of the foreign material may perhaps be detected. Second, the area beneath the curve should diminish, on successive tracings, according to the decay scheme of the isotope studied. If it does not, a radiocontaminant is present.

Two techniques may shorten the counting time required. These are: use of a multichannel analyzer (or counting at the desired photoelectric peak), and the employment of a scintillation well-type counter. The multichannel analyzer rapidly produces the desired scan. If counting is performed only at one photoelectric peak, care must be taken that the response is truly linear at this point; if not, a calibration curve must be constructed. Well-type scintillation counting is readily performed, but it entails the danger of detecting counts from contaminants. Still another approach to the problem is to separate sodium from the other blood constituents, either before or after irradiation.

In addition to sodium, isotopes of chlorine, bromine, iodine, potassium, calcium, copper, magnesium, and possibly cobalt and manganese are produced to the extent of 30 d.p.m. or greater by the exposure of 10 ml. serum to a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for one hour. Quantitative estimation of these materials is theoretically possible if complete separation were possible. Erythrocytes, with a low sodium content, may be directly analyzed by means of neutron activation analysis, and data will be presented in a later report.

SUMMARY

1. A relationship is derived indicating that radioactivity induced in an element common to a standard and an unknown, following identical neutron bombardment, is proportional to the weight of material originally present in each, if correction is made for the time difference between the analyses.

2. Twelve hours after irradiation of the solid material from 10 ml. serum, by a flux of 2.5×10^8 neutrons/cm.^{2}/sec. for one hour, 99.6 per cent of the gamma emissions present are due to Na^{24}.

3. Utilizing the amount of radioactivity in the 12-hour postirradiation specimen, the quantity of sodium present in serum may be estimated. Results agree within three per cent with values obtained by flame photometry.

4. A technique of estimating the area beneath the gamma ray spectral curve of an isotope is described, and its use discussed in detecting radiocontaminants or estimating the quantity of activity.

5. It is concluded that neutron activation analysis may be used to determine sodium in blood serum. The method is essentially nondestructive, and may be performed on samples which must be saved for other determinations. Extensions of the procedure are mentioned, including a technique for the rapid estimation of sodium, and one for the fractionation of serum into its component radioanions and radiocations.

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A WELL SCINTILLATION COUNTER FOR IMPROVED VOLUME EFFICIENCY

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CLEVELAND, OHIO

IN THE use of a well scintillation counter to assay radioactive samples for various clinical tests, it has become increasingly important to employ volumes in the range of 2 to 10 ml. Various techniques involving the determination of plasma levels of protein bound I^{131} after the administration of tracer doses have indicated the advantage of utilizing these larger samples. Two typical techniques used in this laboratory for such determinations include the method of Morton[1] for separating butanol extractable I^{131} from plasma and the method of Lahr and Taberin[2] for separating protein bound I^{131} from the plasma iodide by ion exchange resin. For low level determinations it has been desirable to determine the radioactivity derived from at least a 4 ml. plasma sample, which has entailed analyzing a volume of 8 ml. for the butanol extraction method and 7 ml. when the resin technique is employed.

In addition there are various samples which contain the radioactivity in a nonhomogenous distribution that may vary during the period of assay. An example of this is the measurement of whole blood volume by such agents as iodinated (I^{131}) serum albumin or Cr^{51} tagged red cells. When these whole blood samples are assayed in a well scintillation counter the red cells will, of course, settle to the bottom of the tube while the serum is rising to the top. This presents a continually changing sample geometry to the well counter that can be avoided only by taking short period assays or having a well counter relatively independent of sample volume or distribution.

Since the conventional commercially available thallium activated sodium iodide well crystal exhibits a loss of efficiency for samples much over 2 ml., it would seem desirable to construct a well counter capable of handling larger volumes and exhibiting much less dependency on sample volume or distribution. This has been approximated by the well counter of Baskin, Demorest, and Sandhaus[3] which showed both decreased volume dependency and the ability to handle volumes of 20 ml. or more. This counter, however, is not as efficient as the conventional well crystal until the sample volume exceeds 5 ml. Since many applications will still employ volumes less than 5 ml. there is a reluctance to lose the greater efficiency of the smaller well counter. For this reason a modification in these well dimensions has been made so that the larger well will exhibit the same absolute efficiency as the smaller well, handle larger volumes, and show decreased volume dependency.

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EQUIPMENT

For the larger well we have utilized a sealed thallium activated sodium iodide crystal measuring 2 in. in diameter and 2 in. in height with a well 1⅛ in. in diameter and 1½ in. deep. The outer diameter of this crystal is small enough to be immediately interchangeable with almost all probe units employing RCA 5819 or Dumont 6292 photomultiplier tubes. This well diameter has provided ample room for a brass protective liner in the well while still seating a standard 24 mm. diameter glass vial. The vials utilized were 82 mm. in height and hold approximately 24 ml. These vials[*] are relatively inexpensive and are specified by the manufacturer to be accurate in diameter to within 0.5 mm. Fig. 1 shows the crystal, brass liner, and vials.

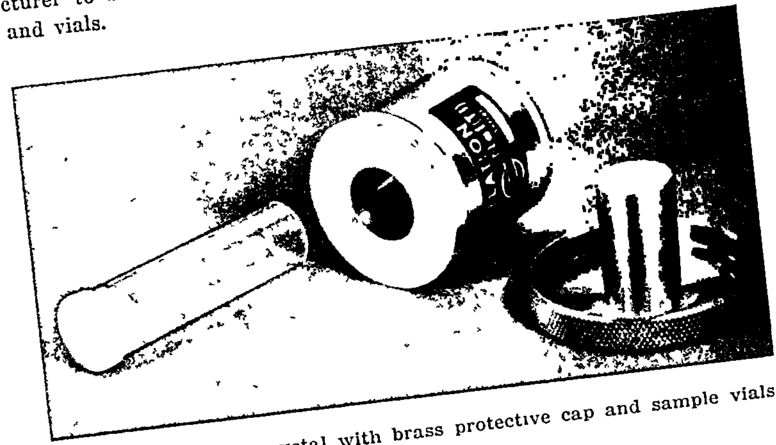

Fig 1.—Large well crystal with brass protective cap and sample vials.

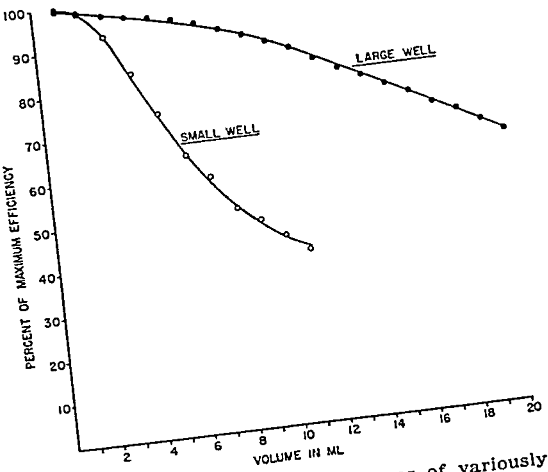

Fig. 2.—Comparison of relative counting efficiences of variously sized well crystals with respect to sample volume. All samples normalized to the response of the small well crystal to a 0/0.01 μc sample of I^{131}.

The conventional thallium activated sodium iodide crystal[†] measured 1¾ in. in diameter and 2 in. in height with a hole ¾ in. in diameter and 1½ in. deep. Klett colorimeter test tubes provide a close fit for this well and have been selected for sample analysis.

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[*] Titeseal vials, The Chemical Rubber Co., Cleveland, Ohio.

[†] Sealed unit provided by the Harshaw Chemical Company, Cleveland, Ohio.

Both well counters were shielded on all sides with approximately 2 in of lead for a total height of 9 in Background of the smaller well counter averaged 250 c p m as compared to 350 c p m for the larger unit The preamplifiers, photomultiplier tubes, and scalers used with each exhibited similar characteristics throughout

RESULTS

A comparison of the relative counting efficiencies of these variously sized well counters with respect to sample volume is shown in Fig 2 The response of the conventional well counter to a 0 01 μc sample of I^{131} in a volume of 0 5 ml has been normalized to 100 per cent The fall off of efficiency as the same activity is assayed in successively larger volumes is then a reflection of both lesser geometric efficiency as well as the self absorption of the gamma rays in the liquid itself

For practical clinical assays the advantages of the larger unit are shown in Table I Relative counting rates as obtained from a 6 ml and an 8 ml whole blood samples from a patient injected with 10 μc of iodinated (I^{131}) serum albumin are shown as counted with both the smaller and larger well crystals

TABLE I COMPARISON OF RELATIVE EFFICIENCY AND VOLUME DEPENDENCY FOR WHOLE BLOOD ASSAY FOLLOWING 10 μC INTRAVENOUS IODINATED (I^{131}) SERUM ALBUMIN ADMINISTRATION

6 ML WHOLE BLOOD SAMPLE	SMALL WELL (C P M)	LARGE WELL (C P M)
Counted immediately	12,600	18,750
After standing ½ hour	11 350	18,450
After centrifuging	8 900	18,200
8 Ml Whole Blood Sample		
Counted immediately	13 850	24 200
After standing ½ hour	12 200	23 700
After centrifuging	7 800	23 000

DISCUSSION

The advantage of improved efficiency is shown in Table I in which it is noted that while only a small increase in counting rate can be obtained by *increasing sample volume from 6 ml to 8 ml in the small well counter*, the larger well exhibits an increase of about one third in counting rate for a similar increase in volume The second advantage observed is the volume independence of the latter unit indicated in the counting rates of the same blood sample after standing 30 minutes and after centrifuging At this time the red cells have predominantly settled to the bottom and the plasma risen to the top, dependent upon sedimentation rate and hematocrit In the patient cited the hematocrit was 39 per cent and the sedimentation rate was 12 mm /hr Since the volume dependence of the small unit is fairly critical this separation has caused a deviation as large as 10 per cent after standing while the larger unit has shown essentially negligible variation

SUMMARY

1 An improved well scintillation counter has been designed for counting radioisotope samples in volumes of 3 to 20 ml with an appreciable increase in efficiency over the conventional well counters used with these volumes

2. The relative volume independence of this unit has indicated its usefulness in producing a stable assay with samples which contain the radioactivity in a nonhomogenous distribution that may vary during the period of measurement.

We wish to thank Miss June Dockery for her assistance in these measurements.

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STUDY OF PAPER ELECTROPHORESIS COMPARISON WITH SALT PRECIPITATION OF SERUM PROTEIN FRACTIONS

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WHEN the Spinco Model R paper electrophoresis apparatus was introduced into our laboratory, we compared this technique with our standard procedure of protein fractionation, a modification of the relatively simple Wolfson and Cohn[1] method In addition, the recoveries of known solutions of albumin and gamma globulin and the "tailing" effect of albumin in the paper electrophoresis system were determined The present paper describes these studies, emphasizing the comparison of the clinical methods of protein fractionation

MATERIALS AND METHODS

Approximately 50 serum specimens were obtained from professional blood donors At the end of the blood donation, approximately 10 ml of blood was collected into a chemically clean test tube the blood was expressed from the tubing through the needle used in the collecting set The specimens were centrifuged and the sera placed in the refrigerator overnight

A control serum was obtained by collecting a unit of clotted blood from one professional donor The serum was separated under sterile conditions and aliquots transferred to sterile, plain Vacutainer tubes† which were stored in a Deepfreeze On the day of collection, control values for both methods of protein fractionation were established by quadruplicate determinations There was no noticeable change in the protein fractions of this control serum when aliquots were later removed and thawed just before using

Both methods of protein fractionation were done in duplicate on each serum simultaneously to avoid possible differences between the two methods, e g , length of time in the refrigerator, temperature changes, and time elapsed between collection and determination We employed the acceptable 6 hour accelerated electrophoretic separation[2] (which compared very well with the usual 16 hour separation) so that both chemical precipitation and paper electrophoresis could be instituted at the same time We added 6 lambda of the serum or protein solution to the paper strips, this volume contained sufficient protein for definite separation and kept the tracing below the 10 cm mark, obviating the use of a factor for calculating the area The paper strips were dyed and fixed according to the method of Block, Durrum, and Zweig[3] After exposure to ammonia fumes[2] the strips were scanned in the Spinco Analytrol Chemical precipitation was accomplished by the Wolfson and Cohn[1] method utilizing the Reinhold[4] modification in which the precipitating solution is a sulfate sulfite mixture instead of sodium sulfite With each series of determinations, an aliquot of the control serum was run in duplicate with each method

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Commercially available human albumin (Cutter) and gamma globulin (Lederle) solutions were diluted with isotonic saline and various mixtures prepared; the total protein concentration was determined by micro-Kjeldahl analysis. Known quantities of the different protein solutions were added to a known serum to obtain the recovery data with the paper electrophoresis apparatus.

RESULTS

Fig. 1 presents the results of the comparison of the two methods of protein fractionation. In each portion of the figure the horizontal, solid lines signify the average value of the specific protein fraction expressed as per cent of the total protein concentration; the dashed lines represent the upper and lower confidence limits or ±2 standard deviations. Each dot is the average of duplicate determinations run on each serum.

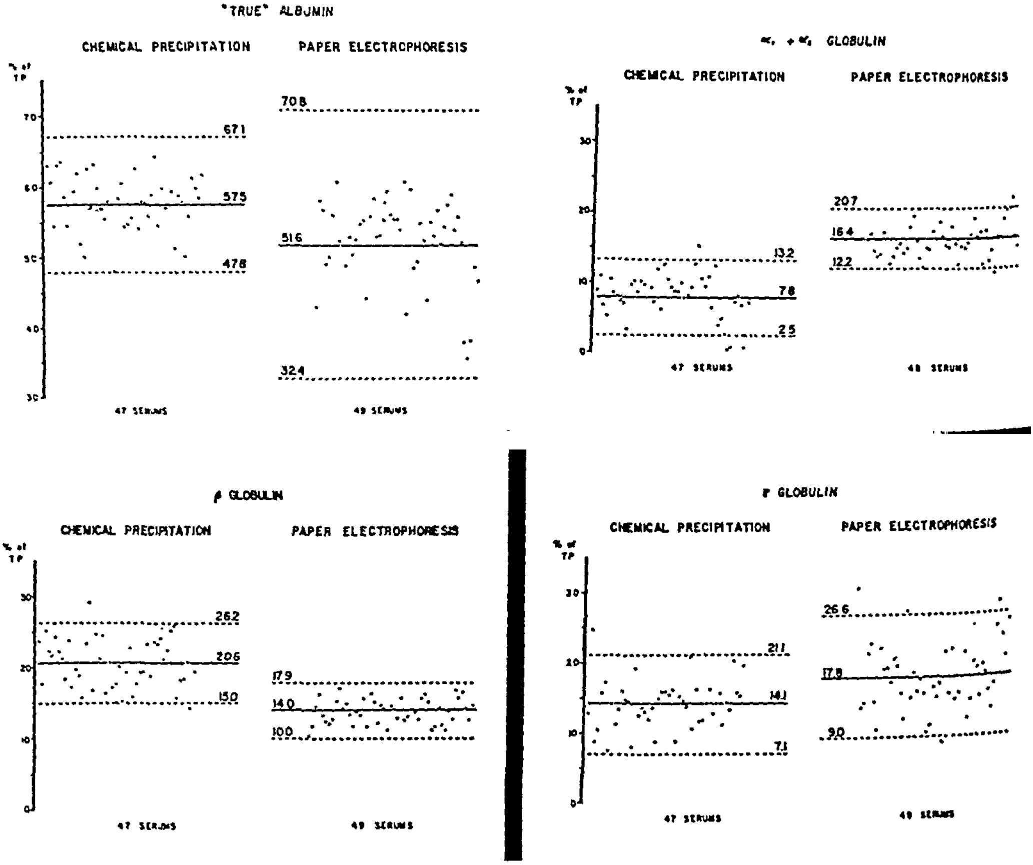

Fig. 1.—Comparison of serum protein fractionation by chemical and Cohn) versus paper electrophoresis (Spinco Model R). Solid hor the average value expressed as per cent of the total protein concen represent upper and lower confidence limits or ±2 standard deviations. Each dot rep the average of duplicate determinations on each serum.

The lower average serum albumin value determined by paper electrophoresis (Fig. 1) may be explained on the basis of the albumin tailing effect; the wider confidence limits may be due to a greater sensitivity of the method and to structural changes in the protein which affect its affinity for the bromophenol blue dye For example, the protein structure may have been altered during the passage of the blood through the tubing and needle of the collection set The average total alpha globulin (Fig 1) is twice as high with the paper electrophoresis method, some of this disagreement may be due to the fact that the alpha globulin is calculated by difference of two separate determinations in the chemical method The average beta globulin (Fig 1) is lower by paper electrophoresis The value for beta globulin in the chemical method is obtained by adding the "true" albumin + alpha globulin and the gamma globulin fractions and subtracting the sum from the total protein, because of this, all the errors which may occur in obtaining the various fractions are summated in this beta globulin fraction

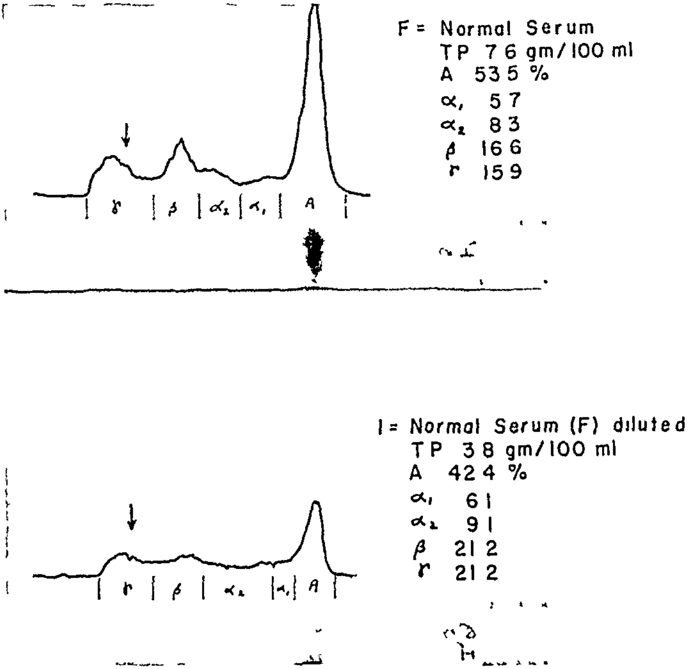

Fig 2—Typical electrophoretic patterns of normal serum obtained with Spinco Model R paper electrophoresis Curve F is undiluted normal serum Curve I is the same serum diluted 1 1 with isotonic saline Line tracing is actual pattern obtained with Analytrol the paper strip dyed with bromophenol blue dye is directly underneath The arrow denotes the point of application of the 6 lambda of solution TP equals total protein concentration in grams per 100 ml A α_1 α β and γ represent the specific albumin alpha 1 alpha 2 beta and gamma globulin fractions respectively in terms of per cent of total dye absorption

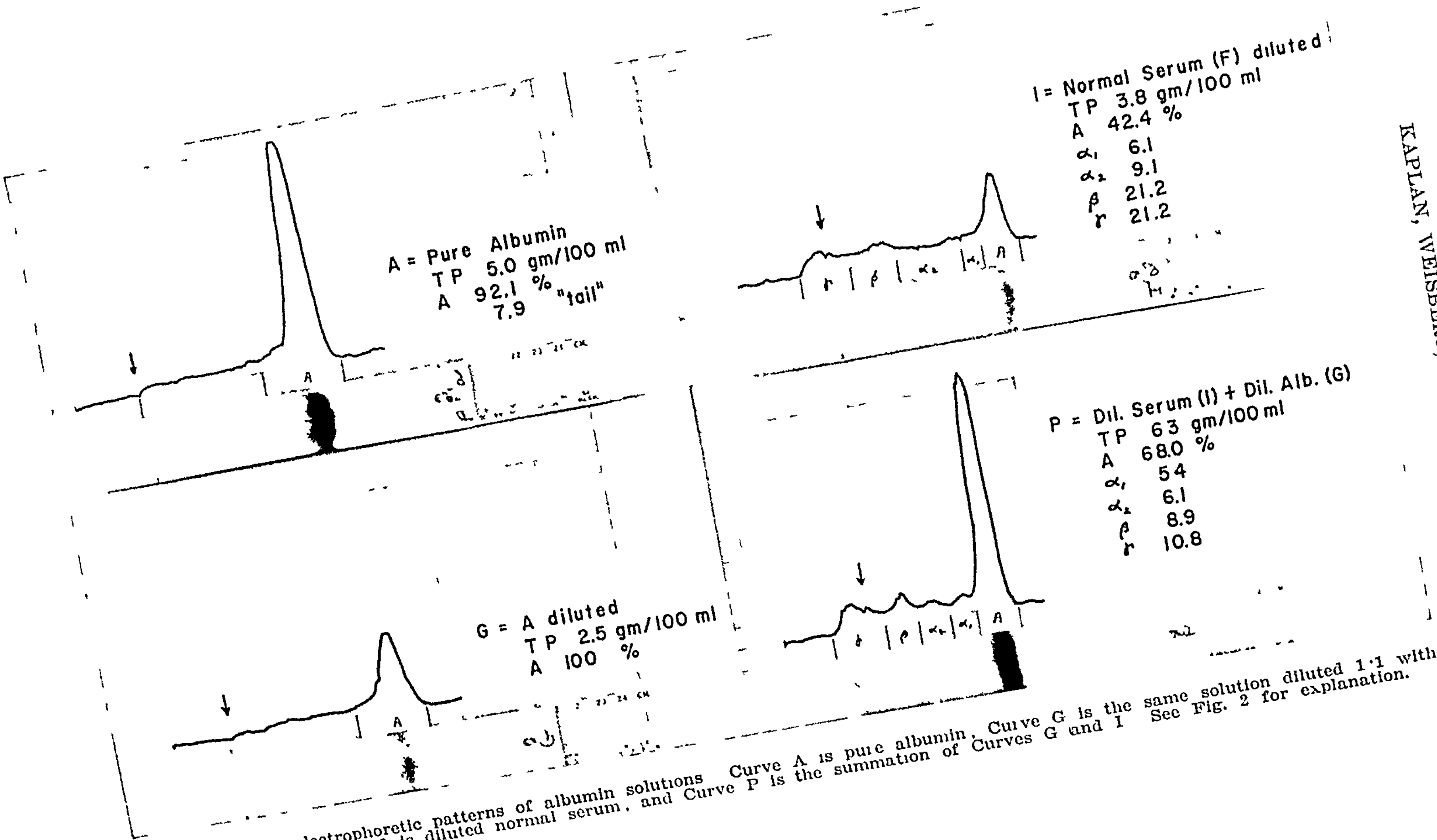

Fig. 3.—Typical electrophoretic patterns of albumin solutions. Curve A is pure albumin. Curve G is the same solution diluted 1:1 with isotonic saline. Curve I is diluted normal serum, and Curve P is the summation of Curves G and I. See Fig. 2 for explanation.

The average for the gamma globulin fraction (Fig 1) is higher by paper electrophoresis The difference may be due to some retention of dye by the albumin tail in the gamma globulin fraction After the strips have been dyed and fixed, we have noted a dark line at the point of application of the sample, this may be denatured protein which does not migrate but does have a certain affinity for the dye, and thereby elevates the gamma globulin concentration in the final calculations

Curve F in Fig 2 illustrates the normal serum used in studying the recoveries of the various protein solutions Dilutions of this normal serum with an equal volume of isotonic saline gave rise to Curve I The low total protein concentration results in a low curve which is difficult to separate into its component fractions, the per cent composition is altered from the theoretical Such alterations are prone to occur in solutions of low total protein concentrations since similar changes in the integration pattern of the Analytrol result in larger numerical changes in the per cent composition

Fig 3 illustrates a study with pure albumin Curve A depicts the albumin solution at a concentration of 5 0 Gm per 100 ml , the tailing effect is calculated to be 7 9 per cent of the total dye absorption as computed with the Spinco Analytrol Even though the line in Curve G is above the base line, nothing was picked up by the Analytrol so that the tailing effect is decreased in the diluted albumin solution with a total protein of 2 5 Gm per 100 ml Curve P illustrates a recovery experiment of adding albumin solution to normal serum This was accomplished by diluting normal serum (Curve F, Fig 2) with an equal volume of pure albumin (Curve A, Fig 3) in essence this can be represented by Curve G + Curve I = Curve P Fig 4 illustrates a similar study with pure gamma globulin, no tailing effect is seen with the gamma globulin solutions (Curves B and H) Fig 5 represents a typical study of one of the different mixtures of albumin and gamma globulin

At a total albumin concentration of 5 0 Gm or more per 100 ml , the tailing effect was found to average 5 7 per cent in 16 experiments When the total albumin concentration was reduced in half, the average tailing for 16 experiments was only 2 8 per cent of the total area This indicates that the concentration of protein in the specimen being analyzed will affect the amount of albumin tailing Controlled conditions regarding albumin tailing and similar integration factors can and should be maintained by applying the same amount of protein (in our experiments approximately 60 mcg) to the paper strip, no matter what the actual protein concentration in the specimen being studied The average recovery, in 16 experiments, of pure albumin added to the normal serum was 90 8 per cent, and 105 2 per cent for gamma globulin

DISCUSSION

Eriksen and associates[2] feel that varying amounts of the different protein fractions are carried over into other fractions during the chemical precipitation, alpha globulin is included with albumin, beta globulin is included with

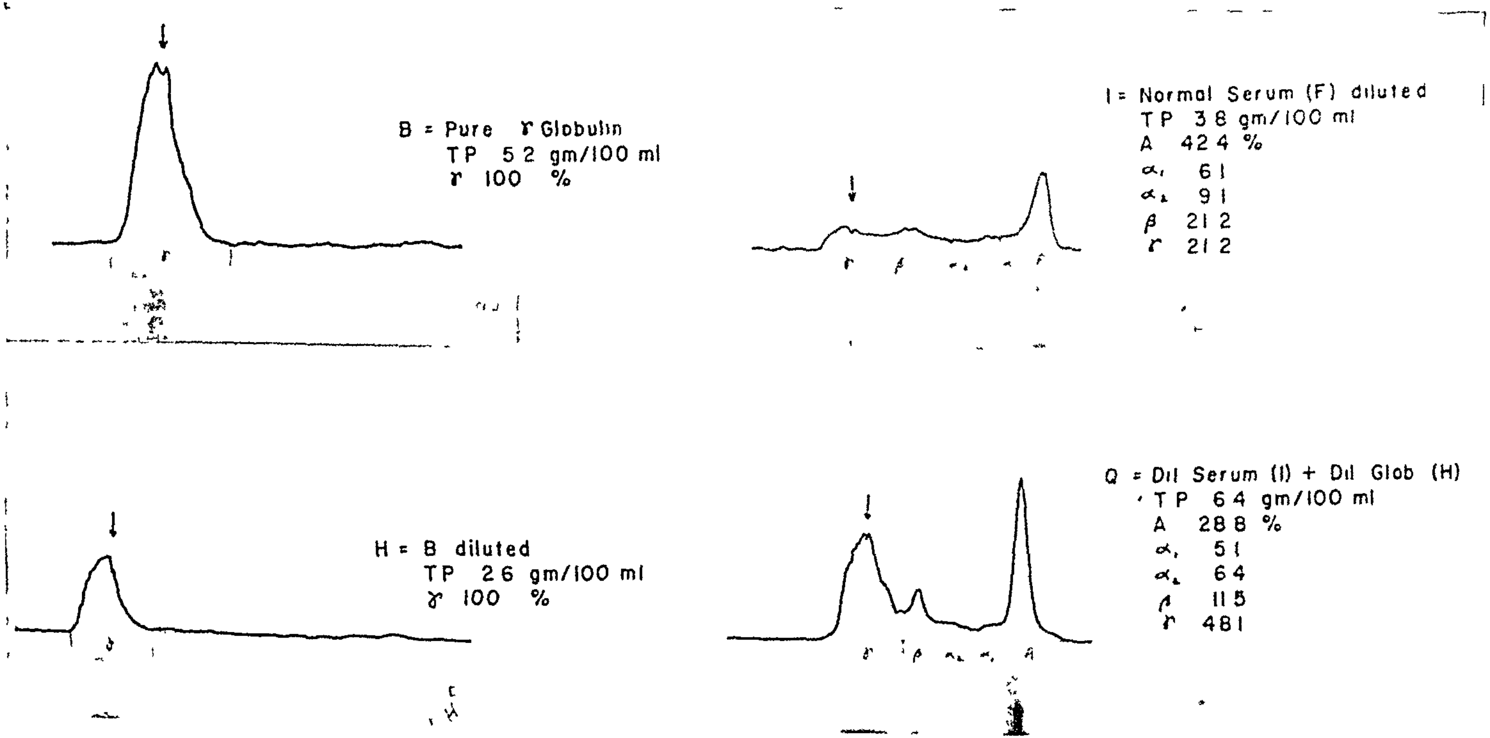

FIG. 1.—Typical electrophoretic patterns of gamma globulin solutions. Curve B is pure gamma globulin; Curve H is the same solution diluted 1:1 with isotonic saline, Curve I is diluted normal serum, and Curve Q is the summation of Curves H and I. See Fig. 2 for explanation.

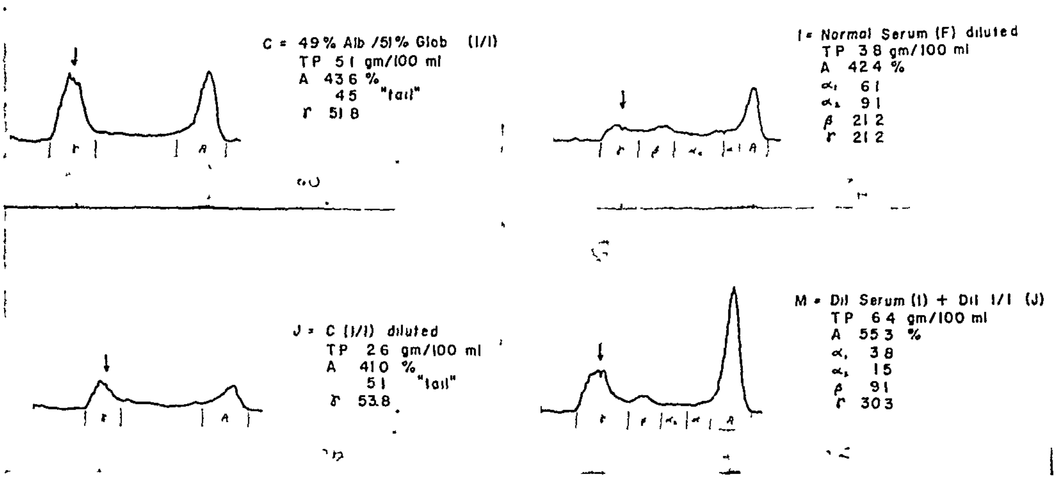

Fig 5—Typical electrophoretic patterns of 1 1 albumin/gamma globulin solution Curve C is 49 per cent albumin and 51 per cent gamma globulin Curve J is the same solution diluted 1 1 with *isotonic* saline Curve I is diluted normal serum and Curve M is the summation of Curves J and I See Fig 2 for explanation

albumin + alpha globulin and with gamma globulin, etc. Thus, the determination of protein fractions by calculation of differences between appropriate quantities may be erroneous because of incomplete separation.

Our results indicate that the two methods of protein fractionation are not interchangeable although there is fairly close agreement between the values for albumin and for gamma globulin. This is not surprising since the two techniques are based on totally different principles—chemical precipitation by salt mixtures versus fixed electrophoresis on paper.

Table I lists representative data for the chemical fractionation of serum proteins by the Wolfson and Cohn[1] method or its modifications and paper and/or free (moving boundary) electrophoresis. The original data of Wolfson and Cohn[1] are based on 4 pools of serum collected from 50 to 150 blood donors. Ellerbrook's[6] larger series of data on normal individuals is in close agreement with our data on professional blood donors. The serum protein fractions by paper electrophoresis show a range of values which may be influenced by the source of donors and the number of specimens studied (Table I). It is obvious that even among the free electrophoresis data there is a wide range of values. The data of Reiner and co-workers[7] illustrate differences in the results obtained on professional versus family blood donors (Table I).

TABLE I. COMPARISON OF REPRESENTATIVE DATA OF PROTEIN FRACTIONATION

INVESTIGATORS	SOURCE* OF SERUM	METHOD OF† FRACTIONATION	NO. OF SPECIMENS	PER CENT COMPOSITION					
					GLOBULIN				
				ALBUMIN	α_1	α_2	TOTAL α	β	γ
Present authors	PD	C	47	57.5			7.8	20.6	14.1
Ellerbrook[6]	N	C	74	58.5			11.1	15.9	14.5
Wolfson et al.[1]	NP	C	4	53.8			15.7	13.4	17.1
Present authors	PD	PE	49	51.6	5.8	10.6	16.4	14.0	17.8
Klatskin et al.[10]	N	PE	10	58.6	3.4	8.0	11.4	12.5	17.6
Knights et al.[11]	N	PE	20	50.0	5.3	9.6	14.9	14.5	20.6
Verghese et al.[12]	N	PE	20	54.0	5.3	8.3	13.6	12.0	20.3
Klatskin et al.[10]	N	FE	10	57.7	4.5	7.2	11.7	15.3	15.3
Gordon[13]	N	FE	37	56.3	5.7	9.3	15.0	14.7	14.1
Reiner et al.[7]	PD	FE	20	53.6	7.5	9.0	16.5	14.1	15.7
Reiner et al.[7]	FD	FE	60	56.8	7.2	8.7	15.9	12.8	14.4
Ricketts et al.[14]	N	FE	10	60.3	4.0	9.7	13.7	12.8	13.2

*PD = Professional Donors. N = Normal Individuals. NP = Normal Pools (50 to 150 donors per pool). FD = Family Donors.

†C = Chemical Precipitation. PE = Paper Electrophoresis. FE = Free Electrophoresis.

Paper electrophoresis and moving boundary electrophoresis do not give exactly similar analytic data[8]; in the latter case the results are expressed in terms of refractive index, whereas in the former they are usually expressed in terms of dye binding capacity, which in the case of acid dyes is largely an expression of the number of basic groups. This dye affinity is subject to many variables, such as method of coagulation of the separated proteins, protein structural changes, type of paper used, etc. The differentiation of the fractions on the tracing made with the Spinco Analytrol, especially for the alpha-1 and alpha 2 fractions, is subjective An electrophoretic peak does not necessarily represent a single, homogeneous protein, but may consist of a group of heterogeneous proteins having a similar electrophoretic mobility The fractions obtained by paper electrophoresis are per cent of the total dye uptake, which we assume to represent the total protein content, we feel that it is erroneous to convert such fractions into terms of Gm per 100 ml All these factors lead us to agree in principle with the work done by Polimeni and Turitto[9] who designate the separated fractions by Roman numerals rather than protein terms We feel however, that for clinical purposes the components should still be called albumin, alpha 1 globulin etc

Because of the conditions of our study—the use of professional donors the method of collection, and the overnight refrigeration of the serum prior to analysis—we feel it is not warranted to list the values obtained by the two methods as normals for serum protein fractionation Rather, we have presented a *comparison* of the results between chemical and paper electrophoretic separation of the serum protein fractions

SUMMARY AND CONCLUSIONS

Our comparison of protein fractionation by the salt precipitation method and by paper electrophoresis showed that the results obtained by the two methods are not interchangeable This emphasizes the fact that each laboratory must always establish normal values for the conditions under which each method is used

The total protein concentration in the specimen to be analyzed by paper electrophoresis is very important because (a) it affects the degree of albumin tailing, (b) the integration patterns with the Spinco Analytrol at low protein levels are not as reliable as those with normal total protein concentration, and (c) low total protein concentration results in poor separation of the globulin fractions

Such conditions should be controlled by adding a relatively similar amount of protein to the paper strip no matter what the actual concentration of total protein in the serum specimen A known serum should be run with each set of unknowns

We wish to thank Dr Kurt Stern and his staff of the Blood Bank at the Mount Sinai Medical Research Foundation and Hospital for their cooperation in obtaining the blood specimens

Thanks are due Mr Richard Bass for technical assistance

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CLINICAL AND EXPERIMENTAL

SITES OF ABSORPTION OF VITAMIN B_{12}

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Although a very small proportion of extremely large doses (500 to 2,000 μg) of vitamin B_{12} can be absorbed, presumably by diffusion, from most mucosal surfaces, the sites of absorption of the much smaller amounts normally available in the diet have not been defined. In the present study, the capability of various segments of the intestine to absorb vitamin B_{12} was assessed by means of the urinary excretion test after Co^{60}-B_{12} was delivered through an intestinal tube.

The absorption of vitamin B_{12} may be determined by 3 general methods: (1) demonstration of a hematologic response in a patient with pernicious anemia in relapse, (2) demonstration of a rise in serum B_{12}, or an increased urinary excretion, as measured by microbiologic assay, (3) measurement of radioactivity involving stools, urine, or serum, or external monitoring of the liver after administration of labeled vitamin B_{12}. Using 100 μg doses, Monto and Rebuck[1] have demonstrated absorption from the nasal mucosa. Ross and his associates[2] and Reisner and co-workers[3] have shown that some absorption of vitamin B_{12} may occur from either the oral or the rectal route, when doses of 500 μg or more are given to subjects lacking endogenous intrinsic factor. Using a test dose of 0.5 μg, McIntyre and his associates[4] found significant

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urinary recovery of cobalt^{60}-labeled vitamin B_{12} after it was introduced directly into the jejunum of 2 patients with jejunostomies. Using timed-release capsules containing 4 μg test doses, Johnson and Berger[5] have likewise demonstrated significant absorption from the lower small intestine of normal subjects.

METHODS

Twenty-one hospitalized subjects were studied as the "normal" group. None had achlorhydria, anemia, intestinal malabsorption, or neurologic or renal disease. Seven hospitalized subjects with Addison's pernicious anemia in remission were similarly studied. None of the patients was seriously ill.

The following modification of the urinary excretion test[6-8] was employed. A test dose of 0.5 μg of Co^{60}-B_{12}* containing 0.25 μc of radioactivity was given to a fasting patient either by mouth or through a tube. Simultaneously, 1,000 μg of nonradioactive vitamin B_{12} was given intramuscularly. All urine was collected for 24 hours, at which time a second intramuscular injection of 1,000 μg was given, again followed by collection of the urine for the next 24 hours. Four hundred milliliters of each sample was counted in a scintillation counter with special shield. Urinary excretion of Co^{60}-B_{12} was expressed as percentage of the administered dose. The range of excretion in normal patients for the two 24-hour collections in our laboratory was 7 to 43 per cent. A third 24-hour urinary collection after 1,000 μg of vitamin B_{12} was given intramuscularly at 48 hours has failed to yield significant urinary radioactivity. In the patients with pernicious anemia, neutralized human gastric juice pooled from 3 or 4 normal individuals was added to the test doses indicated in Table III. Bifacton sec (Organon), an extract of hog's stomach mucosa, which has intrinsic factor activity but contains no vitamin B_{12},† was added to the test dose in other tests.

Eighteen tests were performed by the rectal route of administration in 14 subjects without pernicious anemia. From 0.5 to 3.0 μg of Co^{60}-B_{12} containing from 0.25 to 3.2 μc of radioactivity was instilled through a rectal tube, passed to a level of at least 15 cm. in a patient in the knee-chest position. The subject remained in this position for 15 minutes, and then in the prone Trendelenburg position for one hour. No subject had a bowel movement for at least 4 hours thereafter. The "flushing" doses, of 1,000 μg of nonradioactive vitamin B_{12} were given parenterally at 2 and 26 hours after the instillation. The collection of urine for 2 consecutive 24-hour periods was accomplished as described above. Gastric juice in one test and Bifacton sec in 5 tests was mixed with the test dose. One subject was given twelve grams of neomycin on the day preceding the test. Three subjects were given 250 mg. of tetracycline orally every 6 hours for 4 days before their tests. Tetracycline was likewise given to two patients for 6 and 8 days, respectively, at which time the test dose was administered with intrinsic factor. One dose was delivered to the transverse colon, and one to the cecum, through the intestinal tube described below. Intrinsic factor was added to the dose delivered to the transverse colon.

Intestinal intubation was carried out as follows. A modification of the polyvinyl tube described by Blankenhorn and associates[9] was followed down the small intestine under fluoroscopic and roentgenographic control. Using barium sulfate, with contrasting air insufflation, the pylorus, duodenojejunal junction, and ileocecal valve were used as primary landmarks. The mucosal pattern and the general topography involved were considered as secondary landmarks in identifying the location of the tube. Neither the length of the tube passed, nor the time involved in passage was helpful. If reasonable doubt existed as to the localization, the study was discarded. The effect of barium sulfate on the absorption of Co^{60}-B_{12}, when given orally, was tested in two subjects by adding 50 ml. of a 1:1 barium sulfate suspension to the test dose. The oral urinary excretion test as defined above was repeated 5 times at 7-day intervals in 3 subjects without pernicious anemia. One subject had 4 tests.

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*Generously supplied by Dr. Nathaniel L. Ritter, Merck & Company, Inc., Rahway, N. J.

†Kindly supplied by Dr. Kenneth W. Thompson of Organon, Inc., Orange, N. J.

RESULTS

The reproducibility of the urinary excretion test is shown in Table I Although there are too few tests for statistical analysis, a surprising degree of consistency was obtained in the same subject It is also apparent that barium sulfate had no inhibitory effect on the absorption of vitamin B_{12}

TABLE I REPRODUCIBILITY OF A 2 DAY URINARY EXCRETION TEST IN NORMAL SUBJECTS

TEST*	PER CENT OF 0 5 μG Co^{60} B_{12} SUBJECT 1	2	3	4
1	39 4	30 4	14 0	13 7
2	31 6	28 8	9 9	13 2
3	30 9	29 8	9 7	10 7
4	30 6	27 2	9 2	8 1
5		27 8	9 2†	13 0†

*Tests done at 7 day intervals
†Fifty milliliters barium sulfate mixed with dose

TABLE II URINARY EXCRETION TEST IN NORMAL SUBJECTS

SUBJECT	AGE	PER CENT OF 0 5 μG Co^{60} B_{12} SITE OF DELIVERY OF TEST DOSE STOMACH	DUODENUM	JEJUNUM	ILEUM
G S	30	17 7	39 5		
G	49	24 6	15 6		
H	46	16 4	25 3		
McC	41	13 6	30 2		
O *	57	9 2*	14 7		
D *	58	13 0*	22 2		
W E	60	16 1	26 5	22 8	16 5
D P	39			22 3	
D M S	42				23 2
H B	66	21 1			0†
E J R	33	21 5		12 3	
R W	26				4 7†
R H B	42				18 4†

*Test No 5 in Subjects 3 and 4 in Table 1
†Ileocecal valve visualized

TABLE III URINARY EXCRETION TEST IN SUBJECTS WITH PERNICIOUS ANEMIA

SUBJECT	AGE	PER CENT OF 0 5 μG Co^{60} B SITE OF DELIVERY OF TEST DOSE STOMACH (TEST DOSE ALONE)	STOMACH (TEST DOSE PLUS I F *)	DUODENUM (TEST DOSE PLUS I F)	ILEUM (TEST DOSE ALONE)	ILEUM (TEST DOSE PLUS I F *)
A E	78	0				3 6
G N N	58	2 2	17 6			13 0
P	58	0	6 8			5 7
M A	71	0	13 0		0	
Y	72	0	5 3			4 9
W	66	0	9 7b	6 9b	0	
		3 8	12 6		0	15 3
B	79	0				22 3

*I F = gastric juice 75 to 100 ml (except b = Bifacton sec 30 mg)

Table II shows the results of delivery of vitamin B_{12} by intestinal intubation in normal subjects As can be seen, the recovery following duodenal instillation was significantly greater than in the case of the oral route in 6 of 7 subjects. These differences are especially striking when they are compared to the maximum range of variation found in the reproducibility tests. Significant absorption from the jejunum occurred in 3 subjects and from the ileum in 3 others. Poor absorption from the ileum was noted in 2 additional subjects.

Seven subjects with pernicious anemia were tested with and without intrinsic factor (Table III). Without added intrinsic factor, Co^{60}-B_{12} was not absorbed from the ileum of two patients. With added intrinsic factor, there was significant absorption from the ileum in 6 patients, and from the duodenum in one.

In the 18 tests involving the administration of Co^{60}-B_{12} into the colon, no absorption could be demonstrated in any case. These tests included 6 in which intrinsic factor was added to the test dose, as well as 6 in which antibiotic administration preceded the test dose, given with and without intrinsic factor.

DISCUSSION

By the technique described, rectal instillation probably causes spread of the test dose to at least the level of the splenic flexure. Truelove,[10] by adding contrast material to a rectal drip in a supine patient, has radiographically demonstrated spread of the solution to the splenic flexure and beyond. Forceful injection through a tube in a patient in the knee-chest position followed by the prone Trendelenburg position should, therefore, result in distribution of the test dose at least over the descending colon, and probably to the transverse colon.

It was evident from roentgenograms that the fluid forced under pressure through a narrow bore tube resulted in splattering of the solution over many inches of intestine. Thus it is possible that a portion of the Co^{60}-B_{12} instilled into the terminal ileum might immediately splash into the colon. This might explain in part the low urinary excretion obtained in two subjects, in both of whom the ileocecal valve was visualized within a foot of the tube tip.

The results indicate that in the normal subject vitamin B_{12} in doses of 0.5 μg can be absorbed from the entire small intestine. Inasmuch as intrinsic factor is secreted only in the stomach[11] it is presumed that secretion from that site is available down to the terminal ileum as a result of peristaltic action. Absorption of Co^{60}-B_{12} from the ileum of subjects with pernicious anemia occurred only when intrinsic factor was added to the test dose. Therefore, the ileum is capable of absorbing vitamin B_{12} but only when intrinsic factor is present.

It has been suggested that the process of absorption of vitamin B_{12} normally takes place relatively high in the small intestine.[12] The demonstration that the entire small intestine is capable of absorbing vitamin B_{12} does not invalidate this opinion, for under the normal circumstances of diet and health, vitamin B_{12} is probably absorbed primarily in the more proximal regions of the intestine. The variability of absorption observed from the ileum of normal subjects would appear to be a reflection of either the inconstant availability of endogenous intrinsic factor in the ileum, as a result of destruction utilization, or dilution in its transport from the stomach, or the splashing effect described previously with an immediate loss into the colon of part of the test dose

The inability of the colon to absorb vitamin B_{12}, despite exogenous intrinsic factor, suggests that the mucosa of the colon lacks the necessary transport mechanism The failure of antimicrobial administration to facilitate absorption suggests that bacterial competition or binding in the colon is not the explanation

The striking enhancement in absorption which occurred in 6 of 7 subjects when the stomach was by passed by duodenal intubation is of considerable interest Previous observations have suggested that the stomach may actually play an inhibitory role in absorption Halsted and associates[11] performed fecal recovery tests in 11 patients with a total gastrectomy after gastric juice was added to the test dose of Co^{60} B_{12} In seven, absorption occurred which was much greater than that exhibited either by normal individuals, or by patients with pernicious anemia given gastric juice with the test dose Furthermore, Johnson and Berger,[12] using timed release capsules demonstrated that urinary excretion was greater when Co^{60} B_{12} was released in the small intestine rather than in the stomach

The binding of vitamin B_{12} by proteins without intrinsic factor activity,[14] making it unavailable for absorption, is a possible explanation for an inhibitory role of the stomach However, the effect of pH on intrinsic factor may offer a more likely explanation As early as 1937, Castle and associates[13] described the importance of pH on intrinsic factor extrinsic factor mixtures Beef muscle and gastric juice incubated at pH 2 5 for 12 hours was hematopoietically inert unless partial neutralization to pH 5 was made before administration More recently, from the same laboratory,[15] experiments using gastrectomized rats have shown that incubation of a mixture of Co^{60} B_{12} with homogenized rat stomach at an acid pH reduced the absorption of the vitamin Grasbeck,[16] studying gastric juice by the electrophoretic method, described a striking loss of the main vitamin B_{12} binding fraction (which he speculates may be intrinsic factor) after incubation of the gastric juice at acid pH for longer than 30 minutes If digestion of intrinsic factor should occur after it binds B_{12}, a large portion of the B_{12} might become unavailable for absorption On direct instillation into the more alkaline environment of the duodenum the B_{12} might be preferentially bound to intrinsic factor that has escaped digestion Therefore, it would appear that the destructive effect of an acid environment on intrinsic factor may be the result of proteolytic digestion as suggested by Castle and Ham in 1936[17] This may be a possible explanation for the "inhibitory" effect of the stomach

CONCLUSIONS

1 In doses of 0 5 μg vitamin B_{12} can be absorbed from the entire small intestine provided intrinsic factor is available

2. Absorption of vitamin B_{12} in doses of 0.5 to 3.0 μg from the colon could not be demonstrated in 18 tests despite the addition of intrinsic factor to 6 test doses, and the prior administration of neomycin or tetracycline in 6 tests with or without intrinsic factor.

3. As measured by a 2-day urinary excretion test, the degree of absorption of 0.5 μg of vitamin B_{12} given orally at 7-day intervals was fairly constant in a given individual.

4. Absorption after instillation into the duodenum was significantly greater than after oral administration in 6 of 7 normal subjects. This suggests an inhibitory role of the stomach. Possible explanations for this effect are offered

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FOLIC ACID ABSORPTION, EXCRETION, AND LEUKOCYTE CONCENTRATION IN TROPICAL SPRUE

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INTRODUCTION

FOLIC acid has been widely used in the treatment of tropical sprue since its effectiveness was first demonstrated in 1945.[1, 2] Girdwood[3] has shown that patients with sprue excrete less folic acid in the urine after an oral dose than after a parenteral dose, suggesting impaired absorption or tissue depletion. Garcia Lopez and associates[4] have called attention to the nutritional inadequacy of the diet consumed by patients with sprue in Cuba, but they were able to maintain these patients on a deficient diet and still achieve good hematologic responses with folic acid. However, the roles of dietary deficiency and malabsorption of folic acid remain poorly defined as to the etiology of the disease. For example, a malabsorption syndrome has been observed in military personnel in Puerto Rico who had an adequate diet at their disposal[5] and sprue has been observed in prosperous as well as indigent patients. On the other hand, patients with Whipple's disease or nontropical sprue may show malabsorption without the megaloblastic anemia which is so commonly a feature of folic acid deficiency. It is also curious that sprue is not encountered in certain areas of the world, such as Jamaica, where malnutrition and vitamin deficiency are prominent problems. In considering the role of folic acid in the etiology of sprue, then, the following questions may be asked:

1. Is there an impairment of folic acid absorption from the intestinal contents in patients with tropical sprue?
2. Is intestinal malabsorption the cause or the effect of folic acid deficiency?
3. Is there an intrinsic defect in the absorption of folic acid which produces the disease state?
4. Is the requirement for folic acid higher in patients with sprue than in normal persons?
5. Are there naturally occurring folic acid analogs (e.g., of bacterial origin) which interfere with its utilization? These are some of the questions which prompted the following investigations.

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MATERIAL AND METHODS

Serum and Urine Determinations.—Folic acid was measured by bacteriologic assay using a commercially available medium (Difco), and *Streptococcus faecalis.** Crystalline folic acid was used as the reference standard for the assays and for the oral doses. The dose used for oral administration was 5 mg. of the crystalline material dissolved in 5 ml. of 20 per cent ethanol. For intravenous injection a commercial preparation (Folvite) was prepared so that 10 ml. contained 5 mg. of folic acid. This was confirmed by the assay procedure.

Urine was collected for 5 hours after the administration of a dose. The output was measured and an aliquot was frozen immediately and stored at -20° C. until the folic acid assay could be performed. Storage did not usually exceed 2 to 3 weeks. All subjects were fasting when the dose was administered. In later studies some were allowed to eat a light breakfast 1 hour after dosage since it has been shown that this does not affect the results.[6] Appropriate urine dilutions were prepared and compared with duplicate dilutions of the reference standard. A standard curve was prepared with each group of assays.

Serum determinations were made on samples taken 1, 2, 3, and 4 hours after oral or intravenous administration. Serum dilutions were prepared aseptically and added to sterile media, since it was found that autoclaving the serum mixtures caused protein precipitation which interfered with turbidity readings. Folic acid-free equipment was used throughout in collecting and handling samples.

Leukocyte Assay.—Leukocyte folic acid was measured by a technique similar to that of Swendseid, Bethell, and Bird.[7] For this procedure, 20 ml. of whole blood were collected, heparinized, and mixed in a test tube with 40 ml. of 1 per cent dextran (average molecular weight 193,000) dissolved in 0.85 per cent saline solution. Red cell sedimentation was usually complete in about 2 hours at which time the leukocyte-rich plasma was transferred to a centrifuge tube and spun at 2,000 g for 10 minutes. The supernatant was removed and the remaining cell suspension was centrifuged at 900 g in a Wintrobe hematocrit tube. The height of the leukocyte column was measured and was converted to milliliters by a factor of 0.067 ml. per centimeter determined by calibration of the hematocrit tubes in use. In most cases the red cell volume was less than 4 mm. in height and the platelet layer less than 3 mm. The white cell column varied from 5 to 20 mm. Observations here and in other laboratories indicate that platelets and red cells do not significantly interfere with the results. The concentrated material was diluted 50 times with equal quantities of distilled water and 1 per cent sodium acetate buffer at a pH of 4.5. This was incubated for 18 hours at 37° C. to liberate conjugated folic acid utilizing the naturally occurring conjugase present in leukocytes. The conjugase activity was inactivated by boiling 10 minutes, after which cellular debris was removed by centrifugation. The supernatant was adjusted to a pH of 6.5 to 7.0 with 1 N NaOH and dilutions were prepared for assay as in the other determinations.

Chromatography.—The 24-hour urine collections of 6 patients with tropical sprue *in relapse* and of 2 in remission were concentrated by evaporation to a volume of about 2 ml. and one dimensional chromatography was carried out in a buffered solution of mono- and disodium phosphate at pH 7.0. Spots representing 0.01 and 0.02 of the 24-hour urine volume were used. Strips were cut in half, longitudinally, and assayed by the bioautographic technique described by Nichol, Zakrzewski, and Welch.[8] Folic acid, aminopterin, *a*-methopterin, and leucovorin were chromatographed in 1 μg quantities as reference standards. Fountain and associates[9] have shown that boiling in vacuo does not inactivate *a*-methopterin and we have found that boiling for 3 hours without vacuum does not inactivate *aminopterin*.

Subjects.—Patients referred to as having untreated sprue were diagnosed by the clinical findings of diarrhea, glossitis, and weight loss, together with demonstration in the laboratory of megaloblastic changes in the bone marrow and malabsorption of two or more test substances (xylose, vitamin A, butter, dietary fat). This term was used also for patients in relapse with a similar picture. Treated subjects were those who had previously been studied during relapse, or who gave historical evidence of diarrhea, glossitis, anemia, and who responded to specific therapy. Maintenance therapy consisted of one tablet (5 mg.) of folic acid daily. When treated patients were studied the maintenance dose of folic acid was withheld for one or more days prior to administration of the test dose. Control subjects were laboratory technicians, hospital attendants, or hospital patients without hematologic or gastrointestinal disease.

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*The organism used for this study was *S. faecalis* (29-21) kindly provided by Dr. W. N. Pearson, Department of Biochemistry, Vanderbilt University, Nashville, Tenn.

RESULTS

1. *Urinary Excretion.*—The urinary excretion values in 5 hours after the 5 mg. oral or intravenous dose are summarized in Table I. The difference between the folic acid output of controls and untreated subjects is significant at the 1 per cent level. These results compare favorably with those of Swendseid and associates,[10] who found a normal average excretion of 1.5 mg. in 24 hours (30 per cent of the oral dose) and of Girdwood,[3] who considers excretion of less than 1.5 mg. after a 5 mg. parenteral dose indicative of tissue depletion. The present findings differ slightly from Girdwood's in that treated subjects excreted essentially normal amounts of folic acid whereas his tropical sprue subjects who were described as being in poor condition showed low excretion values even after treatment.

TABLE I. FOLIC ACID ASSAY. MEAN URINARY EXCRETION IN 5 HOURS AFTER A 5 MG. DOSE

SUBJECTS	NO. OF ASSAYS	ORAL			INTRAVENOUS		
		MG.	S.D.	PER CENT OF DOSE	MG.	S.D.	PER CENT OF DOSE
Normal	22	1.31	±0.44	26.0			
	13				2.02	±0.27	40
Treated for sprue	36*	1.21	±0.40	24.0			
	21†				1.97	±0.84	39
Untreated for sprue	10	0.064	±0.071	1.2			
	8				1.39	±0.57	27

*Includes 22 subjects of whom 14 also had a repeat test at a later date.

†Includes 16 subjects of whom 5 also had a repeat test at a later date. In all other cases there was one assay per subject.

TABLE II. FOLIC ACID. MEAN URINARY EXCRETION VALUES IN 5 HOURS FOLLOWING A 5 MG. DOSE

SUBJECTS	ORAL DOSE (PER CENT EXCRETED)	INTRAVENOUS DOSE (PER CENT EXCRETED)
4 untreated patients	0.6	24
3 of original 4 patients after		
1 to 2 mo. treatment	19.0	29
4 to 5 mo. treatment		32

Serial observations were made on 4 subjects before and during treatment. The results are summarized in Table II. It may be seen that within 4 to 8 weeks of folic acid therapy the urinary excretion of folic acid after a 5 mg. oral dose was about 30 times greater than before therapy. The output after intravenous administration improved slowly but had not reached the normal value after 4 to 5 months of therapy.

2. *Serum Levels.*—The serum concentrations after oral administration are shown graphically in Fig. 1.

A "t" test comparing the 2-hour value of untreated subjects with the normal 2-hour value indicates significance at the 1 per cent level.

Serum concentrations were determined after intravenous administration of the 5 Gm. doses to 2 normal persons, 9 treated and 3 untreated sprue patients (Fig. 2). Over 80 per cent of the injected dose was no longer measurable at the end of one hour. It appears that clearance proceeds at a rather rapid rate in each type of subject. There was no trend in this small group of subjects suggestive of an avidity for folic acid in sprue, comparable to that noted with vitamin C in scurvy. It appears that plasma clearance is essentially the same whether the folic acid enters the urine or tissues. Since the method described measures only free folic acid some of the clearance could be due to binding by plasma protein.

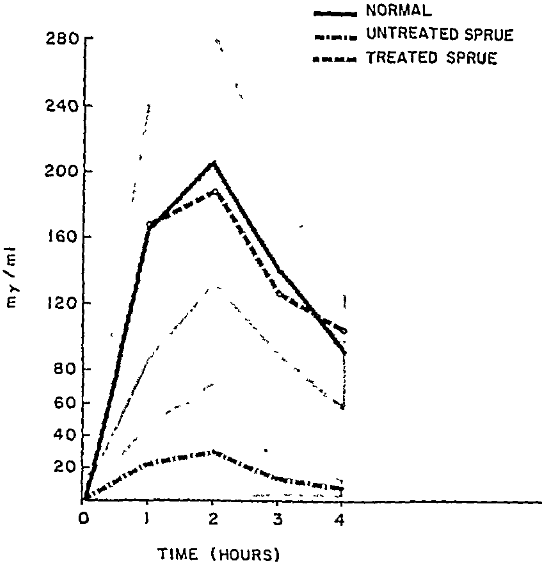

Fig. 1.

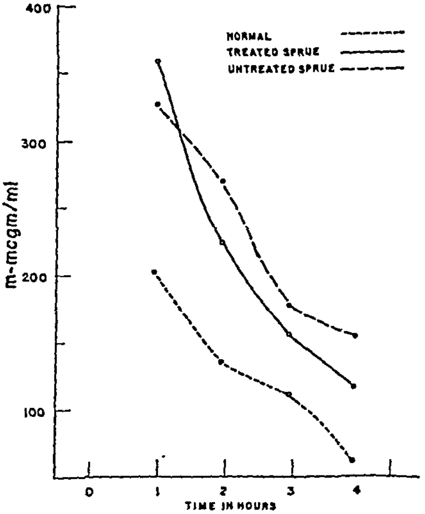

Fig. 2.

Fig. 1.—Mean serum folic acid concentrations at hourly intervals after the oral administration of a 5 mg. folic acid dose to 4 normal subjects, 5 patients with treated sprue, and 6 patients with untreated sprue. The shaded area represents one standard deviation above and below the mean value for normal and untreated subjects. The standard deviation for treated subjects has been omitted for the sake of clarity but is similar to that of the normal group.

Fig. 2.—Mean serum folic acid concentrations at hourly intervals after the intravenous administration of a 5 mg. folic acid dose to 2 normal subjects, 9 patients with treated sprue, and 3 patients with untreated sprue.

3. *Leukocyte Folic Acid.*—The results of leukocyte assay are summarized in Table III. The difference between the untreated sprue group and the normal subjects is significant at the 1 per cent level.

TABLE III

SUBJECTS		WBC FOLIC ACID IN MμG PER ML.		
		RANGE	MEAN	S.D.
Normal	13	46- 97	69.3	15.2
Treated for sprue	6	58-114	81.5	19.2
Untreated for sprue	12	<24- 66	49.1	11.5

4. *Recovery Tests and Chromatography.*—Since the method of urinary assay described does not take into account the possibility that natural antimetabolites are present, recovery experiments were performed in an effort to determine if competition with some inhibitor could explain some of the low levels of urinary folic acid excretion previously noted in untreated subjects. The average amount recovered in 5 normal urines was 115 per cent (range 99 to 126) and in 8 treated patients the recovery was 96 per cent (range 86 to 109). Girdwood[3] has also reported satisfactory recovery of folic acid added to urine. Before untreated subjects were studied it was decided to abandon this method of approach in favor of the more sensitive chromatographic technique.[8]

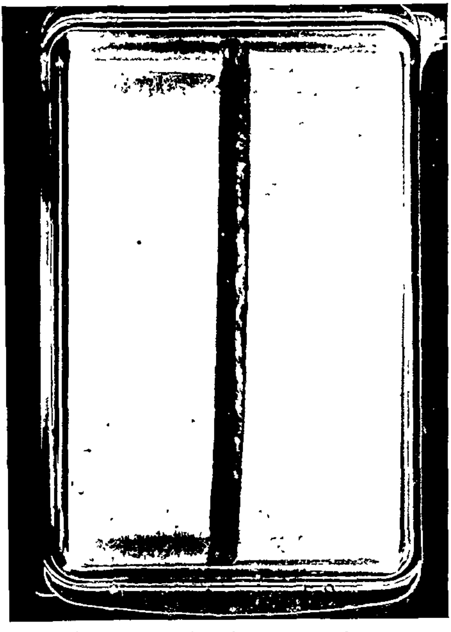

Fig. 3.—Bioautograph of a paper strip chromatogram of aminopterin. The strip was cut in half longitudinally, after which half was applied for 10 minutes to the surface of the folic acid-free medium on the left and the other half to the medium containing folic acid on the right. Growth occurred in the compartment on the left corresponding to the Rf value for folic acid known to be present in aminopterin and inhibition occurred in the compartment on the right at the Rf value for aminopterin

This method permits detection of certain folic acid inhibitors occurring in the same specimen as folic acid, while the recovery technique might fail to reveal inhibition in the presence of an excess of folic acid. The results of chromatography and bioautographs are depicted in Figs. 3 and 4.

therapy, whereas the urinary excretion following oral dosage was approximately 30 times greater than before.

The assay of leukocyte folic acid affords a method of direct observation on cellular stores and serves as a check on the previous findings with oral and intravenous administration. The data indicate a statistically significant decrease in the mean folic acid content of leukocytes in patients with tropical sprue in relapse. However, the fact that the leukocyte folic acid concentration of some patients lies within the lower limit of the normal range tends to support the indirect observations made earlier that tissue depletion is not great. As noted above, patients in relapse retained only 12 per cent more of the intravenous dose than did normal subjects.

Observations were also made on leukocytes from 3 persons with chronic granulocytic leukemia, giving high values, as previously reported by Swendseid and associates. Our highest result was 175.7 $M\mu g$ per milliliter of leukocytes; this was considerably above our normal range but not as high as others have found. It may be of interest that Beard and associates[14] have found abnormally high levels of serum B_{12} in such patients. The white cell folic acid concentration in a man suffering from pernicious anemia in relapse was less than 24.2 $M\mu g$ per milliliter, this being the minimum reading possible with the standard curve. A serum sample drawn at the same time showed a vitamin B_{12} concentration of zero. These findings tend to support the concept of Tasker[15] and others that there may be a correlation between body stores of folic acid and B_{12}.

Several considerations led to a search for possible competitive inhibitors of folic acid. For example, there is some resemblance between tropical sprue and the clinical picture produced by certain folic acid analogs. Thus aminopterin may produce aphthous ulcers in the buccal mucosa, glossitis, diarrhea, leukopenia, and macrocytic anemia.[16] Hamilton and associates[17] have reported that dogs treated with a folic acid antagonist ("DDMP") develop diarrhea, ulcerative lesions in the small intestine, skin pigmentation, and megaloblastic changes in the marrow. Similar results have been noted in human beings.[18] Interest has also been centered on the possible role of bacterial products synthesized in the blind loops, and diverticula of patients with the so-called "pouch P.A.," who respond to antibiotic therapy. Several authors have reported an inhibitor in the plasma of patients with pernicious anemia which interferes with the maturation of megaloblasts in marrow cultures.

The present study failed to demonstrate any substance in the urine of sprue patients inhibitory to the growth of *S. faecalis*. The possibility remains that substances exist which may inhibit growth of human cells and not those of *S. faecalis*.

SUMMARY

1. Evidence is presented that there is marked malabsorption of folic acid in patients having tropical sprue in relapse, and that the tissue levels are moderately reduced.

2. The question remains unanswered as to whether malabsorption is the cause or the effect of folic acid deficiency. However, since folic acid absorp-

tion improves when the absorption of other substances improves it appears likely that this malabsorption may be only a part of intestinal dysfunction

3 Measurement of serum levels after oral and intravenous dosage and measurement of leukocyte folic acid revealed no appreciable differences between normal persons and patients with sprue in remission There does not appear to be a permanent specific defect in the absorption of folic acid by patients with sprue comparable to the malabsorption of vitamin B_{12} by patients with pernicious anemia

4 No evidence has been found that sprue patients in remission require increased amounts of folic acid, other than the well known clinical observations that they tend to relapse without dietary supplements

5 A survey of urines from 8 patients failed to reveal substances inhibitory to the growth of the folic acid dependent organism *S faecalis*

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GLYCOPROTEINS OF NORMAL HUMAN SERUM AS SEPARATED BY ELECTROPHORESIS ON STARCH

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THE pattern of serum glycoprotein following electrophoresis is being increasingly studied as a means of observing serum protein changes in disease.[1-9] At the present time the glycoproteins are visualized either by staining the protein-bound polysaccharides on paper electropherograms with the periodic acid-Schiff (PAS) technique[10] or by determining the content of hexosamines in the separated zones.[2, 3] Such patterns have been evaluated densitometrically and by elution. A lack of specificity[1] and a high background coloration[4] are characteristic difficulties in the paper electrophoresis—PAS method. When glycoproteins are evaluated by their content of hexosamines, determinations are made on the separated zones of paper electropherograms.[2, 3] The paper segments are hydrolyzed and the hexosamine content measured by a modified Elson and Morgan procedure. The most striking characteristic of such data is the absence of a significant amount of hexosamine in the albumin peak; otherwise the data are similar to those obtained by the PAS procedure.

The electrophoretic separation of serum on a starch medium offers the unique opportunity for comparing a glycoprotein curve, measured as hexosamines, with the total protein pattern from the same electrophoretic run. In this paper results are presented for the separation on starch of individual and pooled human sera in an effort to help establish a normal pattern of serum glycoproteins.

METHOD

With the exception of one sample of pooled normal human serum, which had been stored in the frozen state for 6 months, all serum samples were used on the same day as drawn to obviate any changes that might occur either because of storage or the freezing and thawing process.

Zone Electrophoresis.—The technique used here is that of Kunkel.[11] Potato starch,* washed twice with a barbital buffer of pH 8.6 and 0.1 ionic strength, was made into a thick suspension with the buffer and then poured into a plastic mold 15 × 3 × ⅝ inches. After excess buffer was removed with filter paper, the serum sample (3 ml.), previously made into a paste with starch, was packed into a slot approximately 5 inches from the cathodic end of the starch block. Cellulose sponges were used to connect the ends of the block with the electrode vessels. After an initial equilibration period of one hour, 250 v.

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*Amend Drug and Chemical Co., Inc., New York, N. Y., or Fisher Scientific Co., Pitts-were applied which furnished a current of 50 to 70 Ma. The electrophoretic separation as well as the initial equilibration period were conducted at 5° C. After 24 hours the starch was cut into 1 cm. segments each of which was then eluted with 5 ml. of a 1 per cent solution of salt.

Determination of Protein and Hexosamine.—Aliquots of the eluates were analyzed for protein by the method of Lowry.[12] For the determination of hexosamine, 1 ml aliquots were first dried, hydrolyzed for 4 hours with 3 N HCl and dried in vacuo over NaOH. Then the isoamyl alcohol extraction procedure described in detail elsewhere*[13] was used on the neutralized hydrolysates

For total serum hexosamines 1.0 ml. aliquots of a 1:100 dilution of serum were dried, hydrolyzed for 4 hours in 3 N HCl, and treated as described for the determination of hexosamine.

RESULTS

Interference in the recovery of hexosamine by partial hydrolysates of bovine serum albumin using the isoamyl alcohol extraction procedure has been previously reported.[13] To test the effectiveness of this procedure with serum, the recovery of 10 gammas of added glucosamine from 0.01 ml. of human sera was also studied. Ninety-five per cent of added glucosamine was recovered. The absorption curves of the chromogen for glucosamine, serum, and serum with added glucosamine were identical, indicating the absence of interference from nonglucosamine reactive substances of serum.

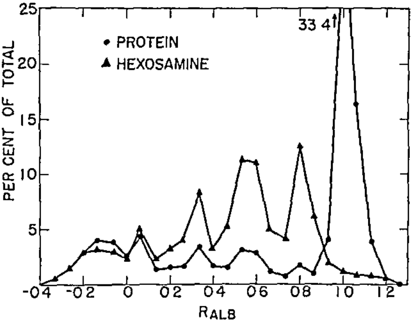

Fig 1.—Glycoprotein pattern obtained by the electrophoretic separation of human serum

The average total hexosamines was found to be 88 mg per cent, a value which is in agreement with that of others.[2, 3, 8] A typical electrophoretic pattern obtained with pooled normal human sera is presented in Fig 1. The protein and hexosamine contents of each segment of starch are plotted as per cent of the total. Each centimeter of starch is expressed as a fraction, R_{albumin}, which is defined as the distance migrated divided by the distance of the albumin peak from the origin. Points anodic to the origin are given positive values; cathodic ones are negative.

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*This technique does not distinguish galactosamine from glucosamine.

The areas bounded by the conventional zones of the protein and glycoprotein curves were measured with a planimeter. The relative proportions of each zone are presented in Table I. No values are given for hexosamine in the albumin fraction as the amount found is insignificant (Fig. 1).

TABLE I. TOTAL PROTEIN AND GLYCOPROTEIN PATTERN OF NORMAL HUMAN SERUM

SERUM	TOTAL SERUM PROTEIN* (GM./100 ML.)	TOTAL SERUM HAm† (MG./100 ML.)	PROTEIN (PER CENT) ALB.	PROTEIN (PER CENT) α_1	PROTEIN (PER CENT) α_2	PROTEIN (PER CENT) β	PROTEIN (PER CENT) γ	HAm† (PER CENT) α_1	HAm† (PER CENT) α_2	HAm† (PER CENT) β	HAm† (PER CENT) γ
Frozen pooled	7.4	94.5	56.6	2.6	9.5	8.5	22.7	25.4	35.7	15.8	23.0
Fresh pooled	7.1	81.3	54.2	5.3	10.2	11.5	18.8	27.2	32.2	22.5	18.1
ON	7.0	81.3	58.5		9.4	10.4	21.7	22.0	31.8	21.0	25.2
GL	7.6	96.3	55.2	5.1	11.1	11.8	16.8	32.1	33.6	18.9	15.4
ML	7.4	73.8	58.0	3.8	10.6	10.4	17.2	24.1	38.6	21.0	16.3
RF	7.9	100.0	52.5	4.0	7.5	10.1	25.9	27.1	29.7	19.9	23.3
AO	8.1	88.7	51.1	5.5	10.7	14.1	18.6	30.0	29.7	23.2	17.1
Average‡	7.5	88.0	54.2	4.7	9.9	11.4	19.8	27.1	32.6	21.1	19.2
s.d.	±0.4	±9.5	±2.6	±1.3	±1.3	±1.5	±3.4	±3.7	±3.1	±1.6	±4.0

*Total proteins were determined by Dr. B. Zak, Department of Pathology, Wayne State University College of Medicine, using a biuret method.[14]

†HAm is used to indicate hexosamine.

‡Data of frozen pooled sera not included.

TABLE II. GLYCOPROTEIN PATTERNS FROM PAPER ELECTROPHORESIS

METHOD	GLYCOPROTEINS (PER CENT) ALB.	α_1	α_2	β	γ	REFERENCE
PAS* densitometric	13.1	17.9	27.9	23.8	17.2	Björnesjö[1]
PAS* densitometric	9.85	15.06	27.27	33.30	14.52	Magalini[6]
PAS* densitometric	25.0	15.0	19.0	21.0	20.0	Grassi[7]
PAS* densitometric	9.8	14.6	29.3	26.8	19.5	Shetlar†[15]
PAS* elution	8.7	21.2	36.0	25.2	8.2	Laurell[4]
Hexose	10.9	13.4	29.4	26.0	20.2	Shetlar†[15]
HAm‡	--	29.2	23.9	25.8	21.1	Goa†[3]
HAm‡	--	20.6	29.5	20.5	29.2	Bollet†[2]

*Stained with periodic acid-Schiff reagent.

†The data of these authors were originally given in mg. per 100 ml. of serum and have been recalculated as per cent of total.

‡HAm represents hexosamines.

DISCUSSION

Available data on the electrophoretic distribution of serum glycoproteins,[1, 4, 6, 7, 15] measured by the PAS stain, exhibit some discrepancies in the albumin and gamma zones (Table II). Some variation is undoubtedly the result of interference by noncarbohydrate substances and individual differences in staining technique. Despite such variabilities the data obtained are in general agreement with glycoprotein patterns based on hexosamine and hexose determinations.[1, 15] With the availability of a simplified analysis for hexosamines the disadvantages of PAS staining can be obviated. The use of hexosamine as a criterion is especially pertinent because of the increasing efforts to follow changes in total serum hexosamine in disease.[8, 9] The data of Bollet[2] and Goa[3] (Table II) combined with those of this paper are a start in developing a clearer picture of the normal distribution of serum glycoproteins as reflected in the distribution of hexosamines.

SUMMARY

Normal human sera were separated electrophoretically using starch as the stabilizing medium. Determinations for protein and hexosamine were made on the consecutive eluates. The average relative distribution of hexosamines in the electrophoretically separated zones was 27.1 per cent in alpha-1, 32.6 per cent in alpha-2, 21.1 per cent in beta, and 19 2 per cent of the total hexosamine in the gamma fraction.

The interest and advice of Drs. A. J. Bollet and B. Zak are gratefully acknowledged.

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ACID MUCOPOLYSACCHARIDE EXCRETION IN THE URINE OF CHILDREN

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URINARY acid mucopolysaccharide (APS) excretion has been measured in normal adults[1, 2] and children[1] and in patients with lupus erythematosus,[3] rheumatoid arthritis,[4] and diabetes.[5] The APS of normal human urine has been shown to be mainly chondroitin sulfate.[6] It was observed that normal children excreted amounts of APS which were several times higher than values observed in normal adults.[1] This report describes a study of its excretion in the urine of normal children, and of children with retarded or accelerated growth.

MATERIALS AND METHODS

Except for a few hospitalized children, the subjects studied were outpatients, followed in the growth and development clinic conducted by one of us (R. M. A.), and normal siblings of a large number of these patients. Each patient had a complete history and physical examination and an x-ray of the wrists for interpretation of bone age.[7] One or more 24-hour urine sample from each patient was analyzed for APS (which is expressed as milligrams of glucuronic acid per 24 hours)[1] and creatinine.[8] The patient or his parents were carefully instructed before, and questioned after, the collection of urine and only those samples which were considered to be complete and to have been continuously refrigerated were accepted. Paper chromatography was carried out in the propanol, phosphate buffer system described by Kerby.[2]

Analyses of the Data.—Notwithstanding a history of complete collection, each sample was examined by an independent means to assess the reliability of the collections. This was done by dividing the patients into 3 groups: those in whom determinations from 2 different urine samples were in close agreement, those in whom the creatinine excretion of a single urine collection was well within the normal range, and those in whom the completeness of the urine collection could not be verified. The first 2 groups were considered reliable.

The entire group studied consisted of 61 male and 26 female children from 3 to 16 years of age. In 49 of these patients, analyses were carried out for APS or creatinine on 2 or more 24-hour urine specimens collected within a period of 6 months. From 39 patients the values were within 15 per cent of their average, and these were accepted as representing complete collections. These patients were designated Group I.

Of Group I, 24 subjects were considered to be normal in that there was no diagnosis of a metabolic or developmental abnormality, and in that the bone age differed by less than 1.5 years from the chronological age. Eighteen of the normal subjects were male and 6 were female. Of the patients in Group I, only the normal males were numerous enough to permit separate statistical analysis. The normal daily creatinine excretion is shown for this subgroup in Fig. 1.

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resent address: Department of Biochemistry, University of Rochester, Rochester, The remaining 38 subjects in this study had only one urine specimen analyzed for APS and creatinine Of the 23 normal males in this group, 17 had values for creatinine excretion which were within one standard error of the normal as determined by the line of regression for the data in Fig 1 These 17 subjects were designated Group II The normal males in Groups I and II were analyzed separately in regard to APS and creatinine excretion related to age, body weight, and surface area They were found to be homogeneous in these respects and were therefore considered together for all further analyses

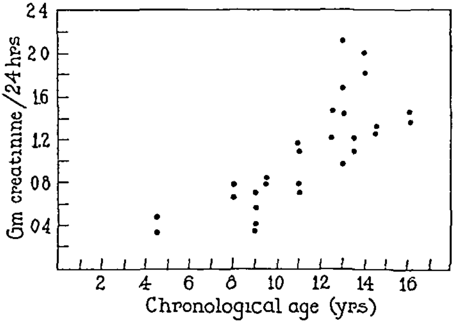

Fig 1 —The creatinine excretion of normal male children

In 31 patients, the accuracy of urine collection could not be verified These subjects were designated Group III. All of the urine collections from patients in Group III were considered to be incomplete, but to represent a major fraction of the 24 hour period

Whenever inspection indicated a linear correlation between a measurement and the subject's age, a line of regression was determined by the method of least squares Standard statistical analysis was employed[9]

Table I The Creatinine Coefficients of Normal Male Children

Age	Creatinine coefficient (mg /24 hr./kg) mean ± S D
<7	14 5 and 19 1
7 8	23 4 ± 4 6
9 10	22 0 ± 2 7
11 12	24 2 ± 8 7
13 14	24 9 ± 5 0
15 16	28 0 ± 4 4

RESULTS

The creatinine coefficient of the normal males was found to vary widely in any age group The mean values at different ages are indicated in Table I It can be seen that the creatinine coefficients of the youngest patients were possibly lower than the normal adult values of 20 to 26 This is consistent with numerous reports of low creatinine coefficients in early childhood[10] However, in the children from 7 to 16 years of age, there was no statistically significant change in the creatinine coefficient with age The few values obtained for females indicated lower creatinine coefficients for this sex at all ages (16 3 ± 2 6 mg /24 hr /Kg)

The 24-hour excretion of APS in normal males and females (Groups I and II) is indicated in Fig. 2. In males there was a progressive increase in APS excretion. This was maximum between the thirteenth and fifteenth year. The highest values of 18 to 23 mg. were 3 times the normal adult value[1, 2] and as high or higher than the values seen in adults with pathologic states associated with increased APS excretion.[3, 4] The values recorded for females were lower than those of males but showed a similar increase during adolescence. This increase occurred, as expected, at an earlier age in female than in male children.

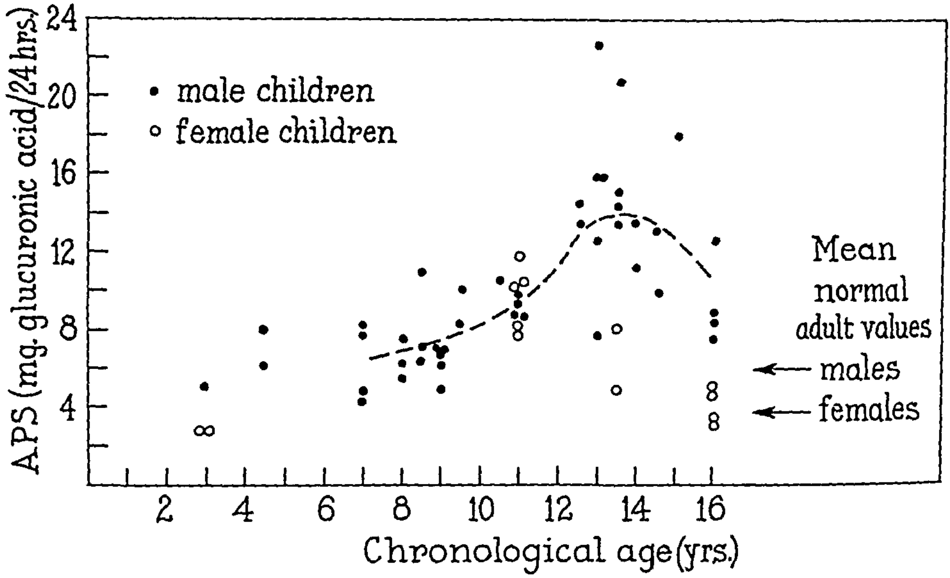

Fig. 2.—The acid mucopolysaccharide excretion of normal children. (The dashed line joins the mean of each 2-year period for male children.)

TABLE II. THE LINEAR CORRELATIONS BETWEEN THE RATIO OF APS EXCRETION TO WEIGHT OR CREATININE EXCRETION AND AGE

GROUP ANALYZED	ABCISSA (X-AXIS)	ORDINATE (Y-AXIS)	LINE OF REGRESSION ± S.E.	COEFFICIENT OF CORRELATION ± S.D.
Normal males Groups I and II	Chronological age (yr.)	$\frac{\text{APS (mg./24 hr.)}}{\text{wt. (Kg.)}}$	y = 1.69 – 0.047 x ± 0.33	– 0.32 ± 0.12
Same	Same	$\frac{\text{APS (mg./24 hr.)}}{\text{creatinine (Gm./24 hr.)}}$	y = 18.8 - 0.73 x ±2.3	– 0.64 ± 0.09
All males (Line for Fig. 3)	Bone age (yr.)	Same	y = 17.9 - 0.71 x ± 2.3	– 0.70 ± 0.07
All females (Line for Fig. 4)	Same	Same	y = 16.7 - 0.76 x ± 1.8	– 0.90 ± 0.04

It is useful to relate any determination from children of different ages to some index to compensate for their continuously increasing body mass. The commonly used indices are weight, height, surface area, and creatinine excretion. The ratio of APS excretion to height or surface area correlated poorly with age When the ratio of APS excretion to weight or creatinine excretion was related to age in this group of normal males, the linear correlations presented in Table II were obtained The calculations from this table

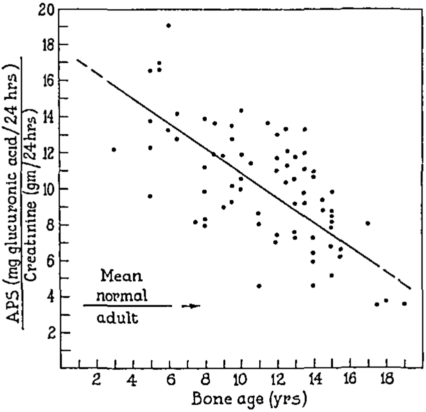

Fig. 3 —The acid mucopolysaccharide creatinine ratio related to bone age of all male children studied (Data from hypothyroid children are not included)

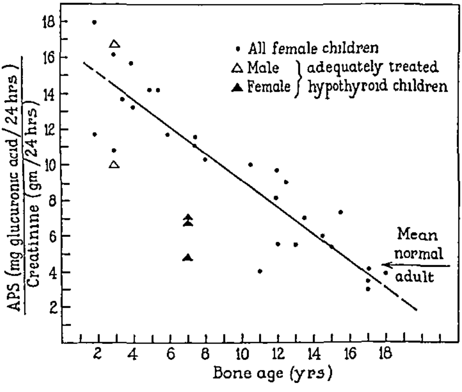

Fig 4 —The acid mucopolysaccharide creatinine ratio related to bone age of all female children studied (Data from hypothyroid children though plotted are not included in the statistical analyses)

show that the correlation of the APS weight ratio with age was of little significance, but that the correlation of the APS creatinine ratio with age was significant As the age increased, there was a regular decrease of the amount of APS excreted per unit of creatinine excreted. This correlation was improved when bone age was used in place of chronological age. The use of creatinine excretion as an index permitted the inclusion in the analysis of patients in Group III.

In Figs. 3 and 4 the APS:creatinine ratio was compared with the bone age for all the males and females, respectively, in the study. There was an obvious difference between the lines of regression drawn from the data from these 2 groups. In both groups the APS:creatinine ratio decreased with age at approximately the same rate, but in females this ratio was lower than in males at all ages. The patients of each sex with advanced or retarded bone age were analyzed separately, and found to show no significant difference from the normal subjects when bone age, but not when chronological age, was used. In every case, the correlation between the APS:creatinine ratio and the bone age was superior to the similar correlation with the chronological age.

The diagnoses other than advanced or delayed growth were enuresis (9 times), dwarfism with or without pituitary or gonadal insufficiency (5 times), alopecia totalis or areata (4 times), hypothyroidism under treatment (3 times). and each of the following once: anorexia nervosa, Osgood-Schlatter's disease, and adequately treated thyrotoxicosis. There was no difference between the APS:creatinine ratio of any of these patients and the others of their sex except in the case of the treated hypothyroid subjects, who had APS:creatinine ratios conspicuously below the values for their ages. (Except in one instance, these ratios deviated from the normal by more than 2 standard errors and none were included in the statistical analyses. Their positions are indicated in Fig. 4.)

The APS isolated from a number of urines which contained large amounts of APS and from several of the younger subjects would not pass by dialysis through a cellophane membrane. Its behavior during paper chromatography and subsequent reaction with toluidine blue was identical to that previously described for APS from normal urines.[1, 2, 6]

It was possible to extend these observations to a very early stage of life because of the interest and enthusiasm of Dr. Helen Ossofsky at the New York Hospital, who made available four 24-hour urine samples collected by catheter from each of 2 infants. A normal male one month old excreted 2.6 ± 0.5 mg. APS glucuronic acid and 0.044 ± 0.006 Gm. creatinine per day, and a 1.5-month old girl with osteogenesis imperfecta excreted 3.2 ± 0.3 mg. APS glucuronic acid and 0.032 ± 0.006 Gm. creatinine per day. The APS:creatinine ratios of these 2 infants were 59 and 97.

DISCUSSION

It has been shown that the urinary APS is not a product of the kidney or urinary tract[2] and further shown that in pooled urine from normal adults and children this APS is mainly chondroitin sulfate.[6] Chondroitin sulfate has also been demonstrated in normal human plasma.[11] The urinary excretion of APS is elevated in several diseases associated with abnormal metabolism of mucopolysaccharide rich connective tissues[3] [4] It is reasonable to suppose that the urinary mucopolysaccharide excretion is a reflection of the metabolic state of the chondroitin sulfate containing tissues of the body

The data presented in this study indicate that there is a regular decrease with age of the amount of APS excreted per unit of creatinine excreted The ratio of APS creatinine excretion attained adult values at about 17 years in females and 21 years in males To the extent that these data reflect tissue acid mucopolysaccharide metabolism they indicate that an initially very active process gradually decreased as the growth rate fell with increasing age, to reach adult levels about the time when growth stopped

SUMMARY

1 Acid mucopolysaccharide (APS) excretion has been described for children of both sexes whose ages were between 3 and 16 years There was a gradual increase in APS excretion during childhood which became maximum during early adolescence

2 The ratio of APS excreted per day to creatinine excreted per day was found to give a linear correlation with age As age increased, a regular decrease occurred in the ratio of APS excretion to creatinine excretion

3 There was no difference in the APS creatinine ratio between normal children and children with advanced or delayed bone age if this ratio were correlated with bone age rather than chronological age

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THE EFFECT OF MORPHINE SULFATE UPON THE RENAL EXCRETION OF WATER AND SOLUTE IN MAN

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THAT the administration of morphine may be followed by a decreased rate of urine formation has been known for a half century.[1] It is commonly stated that this change is mediated primarily by the release of antidiuretic hormone (ADH). Such statements derive largely from studies in which large doses of this alkaloid were administered to experimental animals.[2-9] Observations concerning the effects of therapeutic doses of morphine on the rate of urine flow, glomerular filtration rate, and solute excretion in normal man are so limited and contradictory[10-14] that a study of such effect was undertaken. The data presented demonstrate that morphine, when administered in usual therapeutic doses to healthy men, may decrease the rate of water diuresis without evident release of antidiuretic hormone. Such antidiuresis occurs concomitantly with a reduction in glomerular filtration rate and solute excretion.

METHODS

A water load was established on 22 occasions in 16 normal subjects by the oral administration of 20 ml. of tap water per kilogram of body weight. The water load was maintained throughout the experimental period by providing sufficient water after each voiding to restore body weight to the value attained after the initial loading. The subjects remained recumbent throughout the experiment except when they stood to void. After diuresis had begun, voided urine was collected at intervals of no greater than 30 minutes in all but a few experiments. After at least two periods of "maximal" diuresis, morphine sulfate was administered in a dose varying between 7.5 and 30 mg. subcutaneously or 5 to 10 mg. intravenously. Urine collections were continued for variable periods of time, generally for 105 to 250 minutes after drug administration.

Samples of venous blood were obtained during the height of diuresis and at the end of the study. Blood and urine were analyzed for their content of sodium, potassium, chloride, and creatinine by methods previously described from this laboratory.[15] Total osmolality was determined by freezing point measurement, employing a Fiske osmometer. Inulin and paraaminohippurate clearances were determined in 2 subjects.[16, 17] Equations given by Smith were employed for calculation of osmolal and free water clearances.[18]

RESULTS

1. The central nervous system effects of morphine were apparent within 30 minutes in all but 2 subjects. The manifestations varied in intensity and consisted of drowsiness, "weakness," relaxation, dizziness, and pupillary constriction. In Subject 13 very mild drowsiness and pupillary constriction were

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noted for the first time one hour after the morphine was given. Subject 16 had no subjective symptoms but his pupils became small at the end of an hour. Nine subjects developed nausea at some time during the experiment. Changes in blood pressure did not occur except in one subject who manifested postural hypotension on a single occasion.

2. In 17 of the 22 experiments, within 10 to 55 minutes after morphine administration, urine flow decreased by 2.1 to 6.6 ml. per minute (Table I). In 8 of these 17 the decrease in flow was observed within 30 minutes after morphine was given. The diminution in urine flow was *not* accompanied by a significant rise in urine osmolality. This decrease in diuresis was associated with a reduction in endogenous creatinine clearance varying between 10 and 41 ml. per minute in 16 of the 17 experiments.* In 16 of the 17 studies there was a decrease in total solute excretion varying between 83 and 476 μOsm per minute.

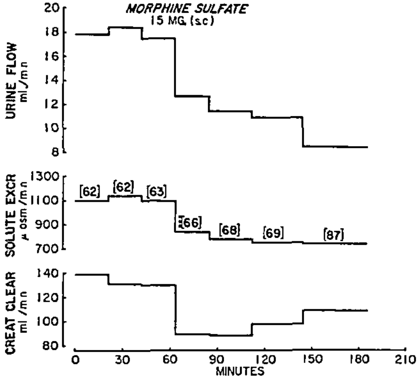

Fig. 1.—The effect of morphine sulfate (15 mg. subcutaneously) upon the renal excretion of water and solute in Subject 3. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

There was little change in the urinary concentration of sodium and chloride when the rate of excretion of these ions decreased with the decline in flow except in one experiment. The urinary concentration of potassium also remained fairly constant when flow diminished, but its rate of excretion decreased in all save 3 instances. As shown in Table I, there was a decrease in osmolal clearances in all but 2 experiments (12a and 12b) and free water clearance generally decreased concomitantly.

The concentration of total solutes and of sodium in serum measured at the height of the initial diuresis and again at the end of the study remained unchanged.

Data illustrating the entire course of 3 experiments are depicted in Figs. 1 to 3. In Subject 3 (Fig. 1) the initial prompt decrease in urine flow was associated with a striking reduction in creatinine clearance and a decline in

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*In Subjects 4 and 8 inulin clearance as well as creatinine clearance was determined and comparable results obtained (Table I).

TABLE I. EXPERIMENTS IN WHICH MORPHINE SULFATE PRODUCED A DECREASE IN URINE FLOW OF MORE THAN 2 ML./MIN. WITHIN 10 TO 55 MINUTES OF DRUG ADMINISTRATION (GROUP I)

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE TOTAL SOLUTE MOSM./L.	URINE TOTAL SOLUTE µOSM/MIN.	URINE SODIUM MEQ./L.	URINE SODIUM µEQ/MIN.	URINE POTASSIUM MEQ./L.	URINE POTASSIUM µEQ/MIN.	URINE CHLORIDE MEQ./L.	URINE CHLORIDE µEQ/MIN.	CLEARANCES C_{Cr} (ML./MIN.)	CLEARANCES C_{Osm} (ML./MIN.)	CLEARANCES C_{H_2O} (ML./MIN.)
1a	40-60	15.7	58	910	4.9	77	10.2	160	9.0	141	140	3.3	12.4
7.5. mg.	60-80*	14.5	60	865	5.1	73	9.7	140	7.8	112	137	3.1	11.4
S.C.	80-105	12.7	58	736	3.8	48	8.8	112	5.5	69	137	2.7	10.0
1b	41-61	22.2	61	1,350	12.4	274	7.4	165	15.8	349	150	4.8	17.4
15 mg.	61-81*	20.4	61	1,240	11.8	241	7.4	151	14.8	301	144	4.5	15.9
S.C.	81-101	15.6	56	874	7.3	114	5.5	85	9.1	142	140	3.2	12.4
1c	40-60	20.9	50	1,040	8.4	176	5.7	120	10.3	214	141	3.8	17.1
15 mg.	60-80*	18.9	50	943	10.0	189	6.3	118	11.8	223	137	3.4	15.5
S.C.	80-100	15.2	54	821	9.6	145	5.9	90	9.6	146	128	3.0	12.2
2a	39-56	18.5	59	1,090	11.5	212	6.4	118	11.0	203	149	4.0	14.5
15 mg.	56-77*	17.6	61	1,070	11.9	209	7.0	123	11.1	196	143	3.9	13.7
S.C.	77-103	14.8	63	933	11.7	173	7.7	113	11.9	176	133	3.4	11.4
2b	45-65	18.5	52	962	7.8	145	4.8	84	7.0	130	149	3.4	15.1
15 mg.	65-76*	17.0	53	906	7.1	120	4.9	84	5.7	96	143	3.2	13.8
S.C.	76-98	14.6	56	827	7.0	104	6.5	97	6.3	92	124	2.9	11.7
2c	160-181	12.1	58	704	10.7	130	3.9	47	11.0	134	133	2.6	9.5
15 mg.	181-201*	11.8	63	740	10.9	128	3.4	40	10.9	128	127	2.7	9.1
S.C.	201-222	9.3	60	553	9.9	92	3.1	29	9.7	90	111	2.0	7.3
3	42-63	17.5	63	1,100	11.3	198	7.0	123	14.7	257	130	4.0	13.5
15 mg.	63-85*	12.7	66	840	13.5	172	7.3	93	18.4	235	89	3.0	9.7
S.C.	85-112	11.5	68	781	12.8	147	7.4	85	18.1	208	88	2.8	8.7
4	20-40	18.5	66	1,226	15.8	292	6.4	119	15.6	288	153†	4.7	13.8
15 mg.	40-60*	16.4	67	1,089	15.4	251	7.0	115	14.8	242	142	4.2	12.2
S.C.	60-80	16.4	63	1,029	14.6	240	7.1	116	13.7	224	151	4.0	12.4
5	40-60	19.7	49	965	5.9	116	6.9	136	6.2	123	143	3.5	16.2
15 mg.	60-81*	17.8	50	896	6.1	109	7.0	125	6.1	108	140	3.2	14.6
S.C.	81-101	14.3	50	719	4.9	70	5.8	84	4.7	67	132	2.6	11.7

6	40 60	14.6	79	1,150	13.4	196	13.8	201	15.8	231	128	4.2	10.4
30 mg	60 81*	12.4	81	953	14.1	166	13.6	160	15.7	184	107	3.5	8.9
S C	81 102	9.0	76	648	10.4	89	11.4	97	12.4	106	91	2.3	6.7
7	40 60	18.4	50	920	4.1	75	8.5	156	7.0	129	150	3.3	15.1
30 mg	60 80*	19.0	51	969	4.4	84	8.8	167	7.5	143	148	3.5	15.5
S C	80 100	16.1	52	837	4.2	67	9.2	148	7.0	112	138	3.0	13.1
8	40 60	15.1	73	1,094	9.6	144	8.5	128	11.6	175	167‡	4.0	11.1
30 mg	60 80*	14.8	70	1 033	10.3	152	7.3	108	10.8	159	163	3.8	11.0
S C	80 100	12.8	73	921	9.0	113	7.3	92	9.6	121	157	3.4	9.4
9a	22 42	18.5	55	1,020	3.1	56	9.6	178	5.1	93	157	3.7	14.8
10 mg	42 62*	14.8	53	772	2.6	38	4.9	73	1.2	62	139	2.8	12.0
I V	62 81	10.0	61	610	0.9	8	8.2	82	2.4	24	141	2.2	7.8
10	61 81	17.5	64	1 122	10.1	178	9.2	161	15.2	266	162	4.1	13.4
10 mg	81 98*	14.3	[illegible]	995	10.1	146	10.7	152	16.9	241	140	3.7	10.6
I V	98 117	14.6	67	975	10.0	146	9.6	140	16.6	243	94	3.6	11.0
11	60 80	13.2	60	789	9.2	121	7.6	100	14.4	189	124	2.8	10.4
10 mg	80 120*	8.5	61	525	5.7	49	7.4	63	10.5	90	105	1.9	6.6
I V	120 180	9.2	60	552	5.2	18	6.3	58	8.6	79	124	2.0	7.2
12a	41 61	19.8	58	1 150	8.4	165	8.0	159	9.1	179	192	4.2	15.6
7.5 mg	61 71*	20.1	59	1,196	9.1	183	8.1	161	9.6	194	197	4.3	15.8
I V	71 96	17.0	62	1,052	9.4	159	9.1	138	10.1	172	182	3.8	13.2
12b	20 40	17.9	56	999	6.7	119	7.5	134	6.8	122	206	3.6	14.3
10 mg	40 70*	16.9	58	986	7.8	133	7.8	124	7.6	131	195	3.5	13.4
I V	70 95	15.1	63	950	9.5	141	7.9	120	10.2	151	184	3.4	11.7

*Morphine administered at the beginning of this period except in Experiments 7, 8 and 9a when it was given at the mid-point of the period

†Corresponding values for inulin clearance were 128, 117 and 126 ml/min. Simultaneous PAH clearances were 681, 616 and 707 ml/min

‡Corresponding values for inulin clearance were 158, 158 and 145 ml/min. Simultaneous PAH clearances were 592, 598 and 507 ml/min

the rate of solute excretion. Approximately 2 hours after the administration of morphine, there was a further decline in urine flow with a rise in osmolality from 69 to 87 mOsm./L. This subject had nausea and vomited at the end of the experiment. Subject 7 (Fig. 2) had a prompt reduction in urine flow associated with a decline in creatinine clearance of 12 ml. per minute and a decreased rate of solute excretion but without a rise in urine osmolality. Approximately 130 minutes after morphine administration there was a striking

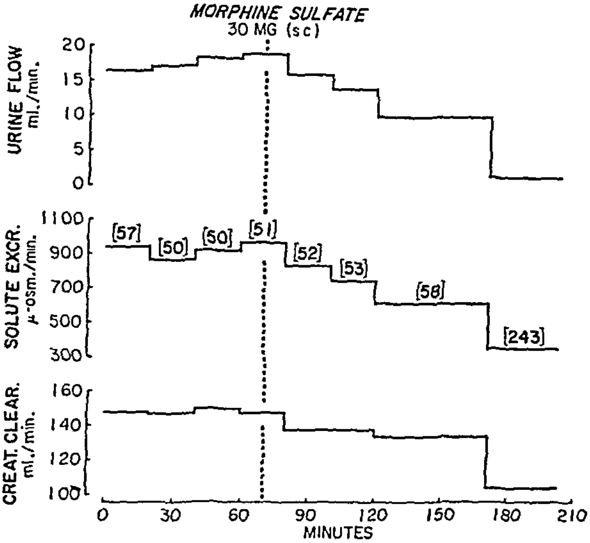

Fig. 2.—The effect of morphine sulfate (30 mg. subcutaneously) upon the renal excretion of water and solute in Subject 7. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

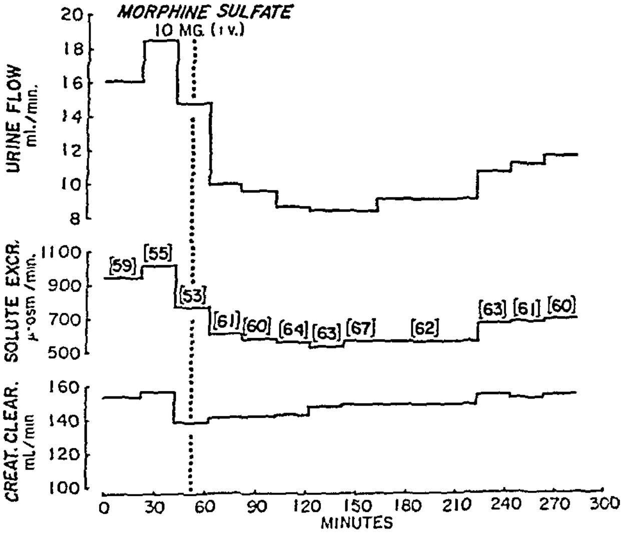

Fig. 3.—The effect of morphine sulfate (10 mg. intravenously) upon the renal excretion of water and solute in Subject 9a. The numbers in brackets are the urine concentrations (mOsm./L.) for the respective periods.

TABLE II. Experiments in Which Morphine Sulfate Did Not Produce a Decrease in Urine Flow of More Than 2 ml./min. Within 55 Minutes of Drug Administration (Group II)

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE: TOTAL SOLUTE: mOSM/L	URINE: TOTAL SOLUTE: μOSM/MIN.	URINE: SODIUM: MEQ/L	URINE: SODIUM: μEQ/MIN.	URINE: POTASSIUM: MEQ/L	URINE: POTASSIUM: μEQ/MIN.	URINE: CHLORIDE: MEQ/L	URINE: CHLORIDE: μEQ/MIN.	CLEARANCES: C_{Cr} (ML./MIN.)	CLEARANCES: C_{Osm} (ML./MIN.)	CLEARANCES: C_{H_2O} (ML./MIN.)
9b	42–64	13.9	53	735	3.7	51	10.1	140	5.8	81	149	2.7	11.2
5 mg.	64–84*	12.8	60	765	2.9	37	10.0	128	3.8	48	152	2.8	10.0
I.V.	84–104	11.9	59	699	2.4	29	9.5	112	3.3	39	149	2.5	9.4
13	20–40	16.4	69	1,134	12.9	211	8.2	135	14.2	233	159	4.1	12.3
10 mg.	40–60*	16.3	71	1,170	14.6	236	7.6	123	15.7	256	169	4.3	12.0
I.V.	60–80	14.7	75	1,095	16.9	249	6.9	101	18.1	269	165	4.0	10.7
14	49–77	11.5	62	715	5.0	57	8.0	92	6.8	79	126	2.6	8.9
15 mg.	77–99*	11.0	62	682	5.6	62	7.9	87	6.6	73	124	2.5	8.5
S.C.	99–128	11.0	61	675	5.6	62	6.8	75	5.4	60	130	2.5	8.5
	128–156	6.1	82	498	7.9	48	7.0	42	6.8	41	113	1.8	4.3
15	25–45	18.2	61	1,100	14.1	258	5.0	91	11.9	218	138	4.0	14.2
15 mg.	45–65*	19.5	65	1,260	16.8	328	5.5	107	14.1	275	135	4.6	14.9
S.C.	65–85	18.8	66	1,240	17.2	324	6.9	129	14.1	264	128	4.5	14.3
	85–106	17.6	72	1,270	18.6	328	7.5	132	15.8	282	123	4.6	13.0
	106–130	18.0	79	1,430	23.3	423	7.5	136	20.2	365	126	5.2	12.8
	130–159	14.0	76	1,060	18.8	263	7.1	99	17.6	245	124	3.8	10.2
16	90–120	9.4	85	796	10.7	100	5.7	54	10.4	98	135	2.8	6.6
15 mg.	120–150*	10.0	83	830	13.0	130	4.9	49	11.7	117	132	2.9	7.1
S.C.	150–210	11.6	80	910	12.9	155	3.9	44	12.2	140	141	3.2	8.4

*Morphine administered at the beginning of this period except in Subject 15 to whom it was administered at the mid point of the perio

TABLE III. EXPERIMENTS IN WHICH A LATE DECREASE IN URINE FLOW WAS ASSOCIATED WITH A RISE IN URINE OSMOLALITY

EXPERIMENT NO.	ELAPSED TIME (MIN.)	FLOW (ML./MIN.)	URINE								CLEARANCES		
			TOTAL SOLUTE		SODIUM		POTASSIUM		CHLORIDE		C_{Cr} (ML./MIN.)	C_{Osm} (ML./MIN.)	C_{H_2O} (ML./MIN.)
			MOSM./L.	μOSM/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.	MEQ./L.	μEQ/MIN.			
1b	41-61	22.2	61	1,350	12.4	274	7.4	165	15.8	349	150	4.8	17.4
	61-81*	20.4	61	1,240	11.8	241	7.4	151	14.8	301	144	4.5	15.9
	81-101	15.6	56	874	7.3	114	5.5	85	9.1	142	140	3.2	12.4
	101-131	13.4	57	764	7.7	103	4.6	62	8.4	113	129	2.7	10.7
	131-162	5.3	135	719	27.0	143	10.6	56	30.4	162	127	2.6	2.7
	162-192	5.2	178	926	37.7	196	11.2	58	40.9	213	138	3.4	1.8
	192-222	11.3	90	1,020	22.1	250	4.6	52	20.5	232	139	3.7	7.6
	222-242	14.4	73	832	19.1	275	4.0	57	17.1	246	141	3.1	11.3
	242-262	15.4	71	1,090	19.3	297	3.2	50	17.6	269	138	4.0	11.4
1c	40-60	20.9	50	1,040	8.4	176	5.7	120	10.3	214	141	3.8	17.1
	60-80*	18.9	50	943	10.0	189	6.3	118	11.8	223	137	3.4	15.5
	80-100	15.2	54	821	9.6	145	5.9	90	9.6	146	128	3.0	12.2
	100-125	8.1	82	663	11.2	91	8.1	65	13.7	111	124	2.4	5.7
	125-155	8.5	92	670	12.9	109	7.6	64	14.0	119	123	2.4	6.1
	155-185	8.4	95	790	16.3	137	7.3	62	17.1	144	124	2.9	5.5
	185-215	11.8	71	840	12.8	151	5.4	64	11.9	141	124	3.1	8.7
	215-235	12.5	70	805	13.4	154	5.0	58	12.9	148	122	2.9	9.6
	235-255	10.2	76	771	14.5	147	4.2	42	13.9	141	123	2.8	7.4
3	42-63	17.5	63	1,100	11.3	198	7.0	123	14.7	257	130	4.0	13.5
	63-85*	12.7	66	840	13.5	172	7.3	93	18.4	235	89	3.0	9.7
	85-112	11.5	68	781	12.8	147	7.4	85	18.1	208	88	2.8	8.7
	112-146	11.0	69	759	11.3	124	7.3	80	15.7	176	98	2.8	8.2
	146-185	8.6	87	750	13.7	118	6.4	55	18.6	160	109	2.7	5.9

5	40–60	19.7	49	965	5.9	116	6.9	136	6.2	123	143	3.5	16.2
	60–81*	17.8	50	896	6.1	109	7.0	125	6.1	108	140	3.2	14.6
	81–101	14.3	50	719	4.9	70	5.8	84	4.7	67	132	2.6	11.7
	101–152†	2.8	142	397	17.6	49	12.2	34	15.9	44	95	1.4	1.4
7	40–60	18.4	50	920	4.1	75	8.5	156	7.0	129	150	3.3	15.1
	60–80*	19.0	51	969	4.4	84	8.8	167	7.5	143	148	3.5	15.5
	80–100	16.1	52	837	4.2	67	9.2	148	7.0	112	138	3.0	13.1
	100–121	14.2	53	750	3.9	55	8.1	114	6.2	87	138	2.7	11.5
	121–171	10.1	58	620	4.9	52	7.8	83	5.7	61	134	2.3	7.8
	171–203	1.5	242	363	35.7	53	31.7	48	31.3	47	114	1.3	0.2
8	40–60	15.1	73	1,094	9.6	144	8.5	128	11.6	175	167‡	4.0	11.1
	60–80*	14.8	70	1,033	10.3	152	7.3	108	10.8	159	163	3.8	11.0
	80–100	12.8	73	921	9.0	113	7.3	92	9.6	121	157	3.4	9.4
	100–120	12.3	71	872	9.9	121	5.6	69	8.6	105	160	3.2	9.1
	120–140	10.9	80	869	11.9	130	5.6	61	9.3	102	160	3.2	7.7
	140–161	4.0	142	573	25.3	103	9.3	38	19.0	77	114	2.1	1.9
	161–202	1.3	390	501	78.0	101	24.2	31	53.8	70	114	1.8	−0.5
14	49–77	11.5	62	715	5.0	57	8.0	92	6.8	79	126	2.6	8.9
	77–99*	11.0	62	682	5.6	62	7.9	87	6.6	73	124	2.5	8.5
	99–128	11.0	61	675	5.6	62	6.8	75	5.4	60	130	2.5	8.5
	128–156	6.1	82	498	7.9	48	7.0	42	6.8	41	113	1.8	4.3

*Morphine administered at the beginning of this period except in Experiments 7 and 8 when it was given at the mid point of the period.

†Experiment 5 was continued for 279 minutes and the urine osmolality increased to 550 mOsm/L, but data are not presented because of incomplete bladder emptying during that entire time.

‡Corresponding values for inulin clearance were 158, 158, 145, 150, 148, 104 and 107 ml/min. Simultaneous PAH clearances were 592, 598, 507, 528, 566, 383 and 400 ml/min.

rise in urinary concentration.[*] Experiment 9a (Fig. 3) is of particular interest in view of the fact that the initial decrease in diuresis was followed by a subsequent return toward the control values. These changes in urine flow were associated with a decline and later recovery of creatinine clearance and solute excretion. There was very little change in urinary concentration throughout the experiment.

3. In 5 studies a prompt reduction in urine flow greater than 2 ml./min. did not follow upon the administration of morphine (Table II). In Experiments 9b and 13 there was a decline in flow of 2.0 ml./min. and 1.7 ml./min., respectively, within 40 minutes after morphine was given, associated with a decrease in solute excretion, but without decrease in creatinine clearance. Urine osmolality rose slightly. Subjects 14 and 15 had a late fall (within 79 and 104 min. after morphine, respectively) in urine flow of more than 2 ml./min. with a decrease in the rate of solute excretion and a reduction in creatinine clearance. In both instances there was an over-all rise in urine osmolality of more than 10 mOsm./L. Subject 16 had a rise in urine flow, increased rate of solute excretion, and a small increase in creatinine clearance without significant change in concentration of the urine.

4. In 6 (Subjects 1b, 1c, 3, 5, 7 and 8) of the 17 experiments, in which the administration of morphine induced a prompt decline in urine flow without rise in osmolality (Table I), there was a later decrease in urine flow of more than 2 ml. per minute. This decrease was associated with an abrupt rise in urine osmolality of more than 10 mOsm./L. and occurred 65 to 143 minutes after morphine administration (Table III). In Experiments 1b and 1c these changes showed definite evidence of reverting toward the control values within 90 minutes. A similar late decrease in flow and rise in urine concentration took place in Experiment 14 in which the earlier decline did not occur (Table III). In 5 of these 7 instances, the patients experienced nausea.

DISCUSSION

The administration of morphine in therapeutic doses to normal men during water diuresis generally resulted in a decrease in the rate of urine flow which cannot be attributed to increased antidiuretic hormone activity in view of the fact that the concentration of the urine did not increase. Although a modest rise in osmolal concentration of a hypotonic urine may not be attributed to antidiuretic hormone effect with complete confidence, unless filtration rate remains the same or increases and the rate of solute excretion remains the same or decreases,[20-22] it is apparent that increased ADH activity cannot be invoked to explain a decrease in flow unless the urine becomes more concentrated. The administration of the alkaloid was followed by a decrease in the rate of solute excretion and reduction in filtration rate, changes that alone or together are sufficient to account for a decrease in the rate of urine flow.[†] In the 7 subjects who demonstrated a late rise in urine osmolality, it is probable that a subsequent release of ADH occurred. It may be noted that 5 of these subjects had nausea which in itself might stimulate secretion of ADH. Since the action of ADH is almost instantaneous, the delayed antidiuretic response cannot be ascribed to an earlier release of hormone."[4]

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[*] The late marked fall in creatinine clearance in this experiment is probably at least in part an artifact due to "dead space error" during an abrupt fall in flow.[19]

[†] On the basis of recent observations in dogs[20, 21] it might be expected that an abrupt fall in filtration rate alone, without the intervention of ADH, would lead to an increase in urine osmolality. Such an association in the present study was not observed even when endogenous creatinine clearance was decreased by over 30 per cent. It is apparent that a species difference may exist. Moreover, even in the dog increased urine concentration in response to lowered filtration rate has not always been observed.[23] Precise definition of factors determining this relationship requires further elucidation.

The data derived from extensive animal studies indicate that under certain conditions morphine acts to stimulate the release of ADH. DeBodo[3] observed unequivocal inhibition of water diuresis in normal dogs who received 2.5 to 5.0 mg per kilogram of body weight of morphine sulfate subcutaneously or intravenously. Further, he showed that an intact neurohypophyseal system was necessary for the antidiuretic action, and that morphine inhibited diuresis even after one adrenal gland was removed while the other was denervated and demedullated. He also demonstrated that morphine did not potentiate the action of posterior pituitary extract in dogs with diabetes insipidus. Duke, Pickford and Watt[25] observed that very much smaller intravenous doses of morphine (0.08 mg per kilogram or less) produced antidiuresis in the dog.[25] This response was abolished by section of the supraopticohypophyseal tract. The final evidence for a direct effect of morphine on the supraopticohypophyseal system under these conditions was provided by the same investigators who demonstrated antidiuresis after 4 to 32 μg of morphine were injected into one or both supraoptic nuclei during water diuresis in chloralose anesthetized dogs. Other investigators have reported that the antidiuresis induced by morphine in animals with intact posterior pituitary gland was associated with the appearance, in the urine of an antidiuretic material which was not morphine and which resembled vasopressin.[3, 5, 7] Nallorphine, which had no effect on diuresis in rats, has been reported to inhibit the antidiuretic action of morphine.*[6, 7] It would thus seem that in the dog the administration of morphine stimulates the release of ADH and it also seems fairly clear that in large doses it has the same effect in the rat.

The possible role of altered renal hemodynamics in the decline in urine flow following morphine administration in animals has also received some attention. Antidiuretic action of the drug has been reported in dogs with diabetes insipidus.[4, 9] Handley and Keller concluded that a dose of 1 mg of morphine per kilogram intravenously in the dog acted only to stimulate ADH while 2 mg per kilogram also resulted in a decrease in filtration rate (creatinine clearance).[4] Crawford and Pinkham[8] also postulated a dual mechanism of morphine action, a "potentiation" of ADH and a renal mechanism, the latter predominant in "hyposthenuric" rats. As indicated above, Duke, Pickford, and Watt[25] observed inhibition of water diuresis by very small doses of morphine unrelated to changes in renal plasma flow or filtration rate.

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*Our observations concerning nallorphine are limited to one experiment. Subject 5 was given 15 mg of the drug subcutaneously on one occasion. His urine flow decreased within 20 minutes from 19.6 ml./min. to 13.4 ml./min. without change in urine osmolality (47 and 48 mOsm./L.) but with a decline in creatinine clearance from 120 ml./min. to 98 ml./min. and a reduction in solute excretion from 925 μOsm/min. to 634 μOsm/min. Forty minutes after nallorphine administration he became extremely nauseated and experienced bizarre feelings that he was unable to describe.

A variety of diverse experiments in human subjects have been reported. The administration of morphine sulfate in therapeutic doses has been reported to cause a reduction in urine flow by a number of investigators.[10-12, 14] In some instances this has been shown to be associated with a rise in urinary concentration of sodium and chloride[10, 14] and a reduction in filtration rate.[11] In one study there was an accompanying decline in paraaminohippurate clearance without change in filtration rate as determined by the clearance of sodium thiosulfate.[12] The study of Kraushaar, Bradbury, Wang, and Brown[11] is of interest in that morphine inhibited the rate of diuresis without increase in urinary specific gravity or urinary chloride concentration. The rate of chloride excretion generally decreased. The same investigators also observed that the administration of morphine to 2 women considered to have diabetes insipidus was followed by a decrease in diuresis, albeit less striking than in the normal. They concluded that increased ADH activity cannot explain the effect of morphine on the rate of urine flow in normal women. DeBodo, commenting on these observations, was of the opinion that the studies did not constitute evidence against increased ADH as the mechanism of the morphine induced antidiuresis and suggested that the 2 women with diabetes insipidus had some residual posterior pituitary tissue.[26] Walker, on the other hand, gave 20 mg. of morphine subcutaneously to 7 normal subjects and observed no change in urine flow despite the presence of nausea and vomiting in some instances.[13] None of these studies in man included measurements of total osmolal concentration of the urine nor was there demonstrated a rise in solute concentration without change in filtration rate. Thus the evidence for ADH release in man following morphine administration is limited.

In contrast to the demonstrated stimulation of ADH release in animals given morphine, it is apparent that in normal man morphine can decrease the magnitude of a water diuresis independent of increased ADH activity. This difference may be attributed to species variation or to the large doses of morphine employed in most of the animal studies compared with the smaller quantities administered to man. However, the latter explanation cannot apply to the observations of Duke, Pickford, and Watt who found evidence for ADH release in dogs after the intravenous administration of morphine in doses as low as 0.08 mg. per kilogram intravenously.[25]

The mechanism responsible for the observed reduction of creatinine clearance and solute excretion after morphine administration cannot be inferred from the data presented. Morphine has little effect on cardiac output, blood pressure, or peripheral resistance in normal recumbent man, although orthostatic hypotension is reported.[27, 28] Blood pressure did not change in our studies. Therefore, it is possible that morphine alters renal function by a direct effect on the intrarenal circulation.

The duration of the alteration in urine flow, solute excretion and creatinine clearance was variable. In one half of the subjects who exhibited a decline in creatinine clearance after morphine, a return toward the control values occurred from 70 to 218 minutes after alkaloid administration, while in the other half no such trend was observed during the experimental period Urine flow did not return to control values during the period of observation, but the role of late ADH release in 7 experiments and the superimposed influence of the diurnal excretory rhythm of water and solute complicates interpretation

The present study was not designed to determine the ability of patients, whose clinical management requires the frequent administration of morphine, to excrete a large volume of administered water during a 24 hour period However, it is apparent that the reduction in urine flow induced by a single dose of morphine in a majority of subjects, would not have compromised their ability to excrete copious urine during a period of several hours More marked impairment was observed only in those instances when pronounced ADH effect was manifest On the other hand, it is possible that in patients who are experiencing pain with consequent ADH release, the administration of morphine by allaying pain may inhibit ADH release and hence promote diuresis *

CONCLUSIONS

1 Morphine sulfate was administered in therapeutic doses, subcutaneously and intravenously, on 22 occasions to 16 normal men undergoing water diuresis

2 In 17 of the 22 experiments, the administration of morphine was followed within 10 to 55 minutes by a decrease in urine flow of more than 2 0 ml per minute The decrease in flow was *unaccompanied* by a rise in urine osmolality, but was associated with a decrease in endogenous creatinine clearance and the rate of total solute excretion

3 In 7 experiments, there was a late decrease in urine flow associated with a rise in urine osmolality

4 Morphine sulfate, administered in therapeutic doses to normal men, may result in a decrease in urine flow without evident release of antidiuretic hormone The decrease in diuresis may be attributed rather to the observed decrease in glomerular filtration rate and in rate of solute excretion

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*One subject was studied under conditions of a single unsustained oral water load After his flow had decreased gradually from a maximum of 20 8 ml/min with an associated osmolality of 57 mOsm/L to a flow of 3 5 ml/min and an osmolality of 307 mOsm/L 15 mg of morphine sulfate were given subcutaneously Within the next 20 minutes there was a further decrease in flow to 1 5 ml/min and a rise in osmolality to 455 mOsm/L However in the subsequent collection period within 55 minutes after morphine urine flow was [illegible] 4 ml/min with a concentration that had decreased to 348 mOsm/L despite an associated decline in creatinine clearance of 13 ml/min In the following periods the urine once again became more concentrated Although the only symptoms presented were related to morphine administration (lightheadedness and unsteadiness) it is possible that the sedative action of the drug resulted in some *inhibition* of ADH release

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COMPLEMENT FIXING ANTIBODY RESPONSE TO M PROTEIN OF NEPHRITOGENIC STREPTOCOCCI IN GLOMERULONEPHRITIS

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THE predominant isolation of type 12 streptococci from patients with acute glomerulonephritis has led to the hypothesis that only certain streptococcal types have nephritogenic properties.[1] If this hypothesis is valid, patients with glomerulonephritis should develop type specific antibodies to streptococcal M protein of nephritogenic types. In an effort to detect such antibodies a series of nephritic sera were examined by the mouse protection and bacteriostatic methods. Although the results clearly demonstrated the occurrence of type specific antibody to nephritogenic streptococci in the sera, it was apparent that technical and biologic factors limited the application of these tests. The protection test required not only a mouse virulent streptococcus, but also large quantities of serum, and the bacteriostatic test was not always reliable if acute phase substance or antibiotics were present in the patient's sera. Because these difficulties could be circumvented with a procedure that did not require living streptococci a complement fixation test was devised using M protein of nephritogenic streptococci as the antigen. An extensive search of the literature revealed no previous report demonstrating complement fixing antibodies to streptococcal M protein in human sera.

MATERIALS AND METHODS

Clinical Material.—The complement fixation test was applied to sera from 3 separate groups. The first consisted of 54 Indian children from Red Lake, Minnesota, with an average age of 5 years, 44 per cent of whom were boys. Serum was obtained from these patients 2 years after their recovery from acute epidemic glomerulonephritis occurring in the Red Lake Indian Reservation of Minnesota.[2] The second group was composed of 52 cases of glomerulonephritis which occurred sporadically in the Dallas area. The average age for this group was 15 years, with 53 per cent of the cases occurring in males. Serum was obtained during the acute illness and for periods up to 36 months following recovery. The final group consisted of 52 nonnephritic adults who served as normal controls.

The diagnosis of acute glomerulonephritis in the patients of group II was suggested in all cases by hematuria. The majority also developed edema, hypertension, a rising ASO titer, or a combination of these features which established the diagnosis beyond a doubt.

Antistreptolysin O.—All ASO titers were determined by the procedure of Rantz and Randall.[3]

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Antigens.—The antigens were prepared by the acid-extraction method of Lancefield[4] from 4 types of Group A streptococci: 3, 40, 12, and the new provisional Red Lake (RL) type.[5] All organisms were typed as such in the laboratories of Dr. Elaine Updyke at the Communicable Disease Center in Chamblee, Georgia. The RL and type 12 streptococci were isolated from patients with acute glomerulonephritis, the RL from a patient in Dallas. The cell sediment from an 18-hour growth in 4.5 L. of Todd-Hewitt broth was obtained by centrifugation, resuspended in 100 ml. N/20 HCl and heated in a boiling water bath for 10 minutes. After cooling, the suspension was neutralized with 1 N NaOH to a pH of 7.2 to 7.4. The clear, yellowish supernatant obtained by centrifugation of the neutralized suspension was stored at 3° to 6° C. after Merthiolate was added to a final concentration of 1:20,000.* Antigenic activity of these extracts remained stable during refrigeration for a period of 4 or 5 months. The extracts were discarded when clouding or contamination rendered them unsatisfactory for use in the complement fixation test.

Antigenic titration was performed with varying dilutions of the extract against a 1:10 dilution of absorbed sera obtained from two patients with acute glomerulonephritis, one following a type 12 and the other a RL streptococcal infection. The type 12 antigen was also titrated against a 1:10 dilution of absorbed rabbit antiserum prepared by the method outlined in *Standard Methods.*[6] The antigenic unit was the smallest amount of antigen preventing hemolysis of sensitized sheep cells in the presence of a positive human serum, 2 full units of complement, and 2 full units of hemolysin.† Two antigenic units were used in the complement fixation test as recommended by Kolmer[7] whose procedure was followed throughout. Because no positive sera were avaibable for titration of the T3 and T40 antigens, undiluted extracts were used in the tests.

Sera.—All sera were absorbed with a streptococcus of a type different than those from which the M-protein antigens were extracted in order to eliminate nonspecific reactions with traces of other antigens which might not have been removed during the acid extraction procedure. Absorption was performed by mixing equal parts of serum and a suspension of packed cells of heat-killed T2 streptococci, usually in the quantity of 1 ml. each. The cells which had been killed by heating for one hour at 56° C. were mixed thoroughly with the serum and incubated at 37° C. for 30 minutes, followed by 18 hours at 3° C.[8] The serum was separated from the cells by centrifugation (5,000 to 6,000 r.p.m.) for one hour. The supernatant serum was transferred to clean test tubes and recentrifuged at the same speed for 30 minutes; this procedure was repeated and the serum then inactivated by heating for 30 minutes in a 56° C. water bath. The repeated rapid centrifugation and complete removal of all absorbing cells effectively avoided the reported tendency for absorption to render serum anticomplementary.[9]

Technique of Complement Fixation Test.—A quantitative complement fixation test was performed according to the method of Kolmer.[10] Since identical results were obtained with the regular and one fifth quantitative methods, the latter has been used more often to conserve serum. Tests were considered negative when complete hemolysis was noted in all tubes; doubtful reactions were recorded when the first tube (1:4 serum dilution) contained a questionable or 1 + turbidity; and a positive test contained at least a 2 + turbidity in the first tube.

RESULTS

Epidemic Nephritis.—Among the unabsorbed sera obtained from the 54 Red Lake Indian children 2 years after the epidemic of glomerulonephritis, 53 fixed complement with a RL extract and 33 did so with the T12 extract. After absorption of 33 of the sera with the indifferent streptococcus, T2, 19 remained positive and 7 doubtful with the RL antigen, whereas 2 were positive and 2 doubtful with the T12 antigen (Table I) The prolonged antibody response in these Indians at Red Lake may be related to the persistence of the RL streptococcus in the epidemic area and the repeated occurrence of subclinical exposures during the ensuing 2 years This is supported by the isolation of the Red Lake streptococcus from the nasopharynx of asymptomatic carriers during cultural studies performed at the time the RL sera were obtained It is also possible that antibiotic therapy may have been insufficient to eradicate the etiologic agent since the average total penicillin dose of 1 3 million units received by the RL nephritic was only 1/10 that given to the average sporadic nephritic in the Dallas area

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*The addition of Merthiolate may raise the pH slightly and neutralization may be required with 1 N HCl.

†Dried guinea pig complement obtained commercially from Hyland Laboratories and Bacto-Antisheep hemolysin from Difco Laboratories were used in all tests after first titrating the exact amount to be contained in two full units.

TABLE I RESULTS OF C F TEST USING UNABSORBED AND ABSORBED SERA FROM 33 RED LAKE INDIANS TWO YEARS AFTER THEIR RECOVERY FROM ACUTE GLOMERULONEPHRITIS

PATIENT	ASO	RED LAKE ANTIGEN		TYPE 12 ANTIGEN	
		UNABSORBED	ABSORBED SERA	UNABSORBED	ABSORBED SERA
A S	250	444300*	43±000	442000	-
W S	125	444000	-	±	
M A G		432000		444200	41±000
L G	333	444400	3±0000	444430	
P G	166	444000	+	421000	
G H	333	4444±0	300000	432100	
J M	333	444400	300000	444100	
E M	125	444400	400000	3+0000	
L D	50	442+00	±	-	
A D		444000		-	-
J G	100	442000	442000	441000	400000
S D	250	442000	-	-	-
E S	166	444300	411000	-	
B W	166	43±000		-	-
M H	250	441±00	-	-	-
B R	100	444400	4±1000	22±000	-
M H	500	444400	3+0000		-
D D		442000	400000	±	±
W G	100	443000	411000	-	
H L	250	444100	4±0000	443000	
B S	166	440000	±	200000	-
H S	100	444100	+	444300	
J S	166	444000	320000	4±0000	
J K	333	444400	±	411000	-
A K	166	444100	±	41±000	-
J R H	333	444400	441000	433100	±
J A L	250	444300	400000	±	
V G	166	442000		400000	
J G	100	443±00	4+0000	444100	
W G	333	444400	443000	444200	
R G	100	444000	±	-	
D H	100	444400	410000	444400	
F D	100	444200	430000	41±000	

*1 2 3 4 = 1+ 2+ 3+ or 4+ complement fixation the series of numbers represents the reactions in serial dilutions of sera beginning with 1 4

Sporadic Nephritis—Unlike the Red Lake sera, the absorbed sera from the 52 Dallas nephritics reacted as frequently with the T12 antigen as with the RL antigen (Table II) Eleven sera gave a positive reaction when tested with either antigen and 13 sera which were positive with one antigen gave a doubtful

TABLE II. RESULTS OF C. F. TEST USING ABSORBED SERA FROM 52 DALLAS NEPHRITICS

PATIENTS	ASO	DAYS AFTER ONSET OF NEPHRITIS	RED LAKE ANTIGEN	TYPE 12 ANTIGEN
E. P.	500	10	3±0000	442000
E. B.	50	425	2±0000	±
M. S.	166	16	200000	42±000
O. S.	333	540	±	-
W. C. A.	100	995	-	-
C. V.	625	60	441000	-
A. C.	650	31	4±0000	±
J. F.	125	100	43±000	±
D. J.	500	9	3±0000	420000
O. D.	1,250	28	300000	440000
H. W.	333	14	±	±
M. B.	625	22	±	±
L. C.	333	940	-	-
P. A.	333	26	-	-
R. L. T.	100	540	-	213000
F. R.	125	510	±	210000
J. M.	125	11	±	±
S. W.	125	300	-	-
J. J.	333	10	-	-
E. J.	500	17	-	-
P. R.	333	6	200000	400001
L. R.	625	6	4±0000	±
F. R.	100	58	200000	4±0000
J. W.	250	5	-	-
J. D.	166	21	-	-
D. D.	166	14	±	-
S. L.	625	20	±	-
B. H.	333	14	-	-
C. S.	333	32	±	±
V. B.	125	21	-	-
F. H.	833	18	4±0000	210000
O. M. E.	333	23	±	42±001
M. F.	2,500	18	43±000	444400
J. S.	500	46	-	-
L. H.	250	44	-	-
J. R.	625	18	444300	444440
E. R.	625	6	±	421000
B. C.	333	18	-	300000
J. M.	2,500	29	444100	444440
J. D.	500	87	±	-
J. D. C.	333	28	±	200000
O. M.	333	14	200000	400000
L. W.	125	6	±	±
M. S.	100	14	-	-
K. S.	250	90	-	-
W. B.	250	109	-	-
B. R. L.	625	57	±	200000
A. P.	333	90	2±0000	-
S. F.	250	51	±	200000
L. L.	250	15	-	-
J. W.	250	12	±	200000
F. S.	625	21	-	420000

reaction to the heterologous extract. This marked overlap in reactivity obtained with these two antigens led to the speculation that a common antigenic substance may be shared by those streptococci having nephritogenic properties. In order to decide which of the two streptococcal types initiated the infection serial tests were done in 5 patients over a period varying from 2 weeks to a year and a comparison was made of the duration and titer of the antibody response to the 2 nephritogenic streptococci in a given patient (Table III) The results demonstrated that one type specific antibody consistently predominated in the sera of a given patient over a period of time The streptococcus to which this dominant antibody corresponded was regarded as the etiologic organism

TABLE III SERIAL C F TESTS USING ABSORBED SERA FROM 5 DALLAS NEPHRITICS

NAME AND SERUM DATE	ONSET DATE AND ASO TITER	TYPE 12 ANTIGEN	RED LAKE ANTIGEN	REMARKS‖
E P	9 3 55			
10 3 55	250	432000	3±0000	Mouse protective
10 13 55	500	442000	3±0000	and bacterio
10 26 55	250	42±000	3±0000	static antibodies
11 4 55	333	42±000	100000	to type 12
12 14 55	333	410000	310000	
4 25 56	166	210000	200000	
7 11 56	250	210000	100000	
10 10 56	250	110000	100000	
M S	12 14 56			
12 21 56	166	3±1000	+00000	Mouse protective
12 27 56	166	3±0000	100000	and bacterio
12 29 56		42±000	200000	static antibodies
1 4 57	250	420000	100000	to type 12
C V	2 3 55			Red Lake strepto
3 21 55	333	000000	441000	coccus isolated
4 13 55	625	000000	44+000	from throat
4 21 55	166	000000	4±0000	Mouse protec tive and bac teriostatic anti bodies to RL
D J	2 9 56			
2 16 56	500	420000	3±0000	Mouse protective
3 3 56	333	444000	+00000	and bacterio
3 12 56	625	441000	100000	static antibodies
7 18 56	250	±00000	+00000	to type 12
O D	6 19 56			Mouse protective
7 11 56	625	410000	000000	and bacterio
7 18 56	625	420000	200000	static antibodies
7 25 56	1 250	440000	300000	to type 12

TABLE IV RESULTS OF C F TEST USING SERUM FROM A DALLAS NEPHRITIC ABSORBED WITH BOTH T2 AND T12 STREPTOCOCCI

(Serum was obtained 6 weeks after onset of sore throat when ASO titer was 625)

PATIENT J R	TYPE 12 ANTIGEN	RED LAKE ANTIGEN
Serum Absorbed with T2	444440	444300
Serum Absorbed with T12	000000	430000

The cross reactivity between the two antigens with a given serum raised the possibility that the RL and the T12 antigens might be the same rather than related The individuality of the two antigens was established by demonstrating the persistence of the RL antibody after removal of T12 antibody by absorption with T12 streptococcal cells (Table IV)

Reactions With T3 and T40 Extracts.—Absorbed sera from nephritics were tested with M-antigens of T3 and T40 streptococci. Of the 21 sera tested with T3, one was positive (1:4) and two doubtful. All 3 sera which showed this weak reactivity with the T3 antigen were strongly positive when tested with the T12 antigen. Twelve nephritic sera tested with the T40 antigen all gave negative results.

Normal Persons.—Among sera from 52 nonnephritics, 18 of whom had ASO titers ranging from 250 to 1,250, 2 contained antibody against the RL antigen, and only one against T12. The titers of the positives were only 1:4. There was no overlap in reactivity among the positive sera.

DISCUSSION

The concept that acute glomerulonephritis is a result of streptococcal infection has recently evolved to the point where only a limited number of streptococcal types are considered to possess nephritogenic potentialities. The evidence in favor of the existence of only a few types of nephritogenic streptococci is based mainly on isolation of these streptococci from patients, and suffers from the limitation that in nonepidemic areas cultures of many patients with nephritis are either negative or yield nontypable streptococci. Among 40 Dallas cases of sporadic glomerulonephritis in this study, for example, streptococci could be isolated from only 20; and of the 14 subjected to typing studies, 10 were nontypable, 3 were T12, and 1 was type RL. The complement fixation test used in this study has been of value, therefore, not only in providing further evidence of infection by those streptococci which have been implicated in glomerulonephritis, but also in extending it by serologic means to include the culturally negative nephritics. While the main objective in this investigation has been to provide a serologic method for obtaining such evidence, the complement fixation test can also be used to help identify those cases in whom the diagnosis of streptococcal nephritis is not clear on clinical grounds. There is a striking lack of type-specific antibody to types 12 and RL in nonnephritics with high ASO titers despite the fact that 12 predominates among the typable streptococci isolated from streptococcal infections occurring in nonnephritics in Dallas.

This difference in type-specific antibody titer in the sera of nephritics and nonnephritics might represent a greater tendency for nephritics to react immunologically to the M-antigens, but more extensive study would be needed to answer this question. Of greater immunologic importance, however, is the finding that nephritics apparently possess antibody to the M-protein of more than one nephritogenic type. This would imply an immunologic relationship between the nephritogenic M-substances which can be demonstrated with immune sera of infected persons but not of animals inoculated with dead streptococci. It remains to be determined whether or not this serologic difference depends on an antigenic response unique to infection or to species differences (i.e., man versus animal), but it is evident that a common property exists which could account for the ability of certain streptococci of different types to induce the same clinical disturbance Because this common feature is antigenic, the possibility exists, therefore that the mechanism of renal injury involves an immunologic process such as hypersensitivity

CONCLUSIONS

1 A complement fixation test has been devised for detecting type specific antibodies to streptococcal M protein

2 The results obtained by application of this test to sera from the Red Lake Indian children indicates that the Red Lake streptococcus is a separate nephritogenic type, immunologically different from type 12 is widely distributed geographically, and evokes a prolonged immunologic response which provides evidence by complement fixation of past nephritogenic infection

3 The data obtained by application of this test to the Dallas nephritics suggest that nephritogenic streptococci contain a related antigenic substance other than the conventionally measured type specific substance, which promotes a prolonged, but not indefinite, antibody response

4 The lack of reactivity noted when normal sera with high ASO titers were tested with the type 12 and Red Lake antigens supports the specificity of the test

5 The paucity of positive reactions obtained by testing the nephritic sera with the T3 and T40 extracts provided further evidence supporting the type specificity of the C F test

6 The nephritics whose sera gave negative results with both T12 and RL antigens may have had an infection with one of the other so called nephritogenic types not included in this study

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AGENTS ISOLATED FROM PATIENTS WITH BOSTON EXANTHEM DISEASE DURING 1954 IN PITTSBURGH

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AGENTS cytopathogenic for tissue cultures were recovered from feces of patients with an unusual exanthematous disease which occurred during 1951 in Boston.[1] More recently another outbreak of a similar illness, for which the term Boston exanthem disease has been proposed, occurred in Pittsburgh during 1954.[2] The disease was observed as a sharp localized outbreak in a suburban community, and also in scattered cases from 4 different areas of the city. Agents cytopathogenic for tissue cultures were isolated from specimens of 12 patients in the community outbreak and from 6 of the scattered cases.

This paper presents additional laboratory studies, not previously reported, which are pertinent to the recovery of these agents from exanthem patients in Pittsburgh, with reference to optimum specimens and tissues for their primary isolation, and tests for neutralizing antibody. Also presented are results from attempts to isolate similar agents from patients with other illnesses, and the findings of a survey for neutralizing antibodies.

MATERIALS AND METHODS

Stool specimens were stored in the refrigerator for 1 to 7 days, or frozen at −15° C. for periods of several weeks to 6 months before preparation as 10 per cent suspensions in phosphate buffered saline. The suspensions were clarified by centrifugation at 8,000 to 10,000 r.p.m. for 30 minutes in the cold. Throat and mouth washings were collected in modified medium 199[3] and neutralized with $NaHCO_3$. Blood clots were ground as 50 per cent suspensions with sterile alundum in phosphate buffered saline within 24 hours after blood was drawn. Pharyngeal washings and suspensions of blood clot were centrifuged at 2,000 r.p.m. for 20 minutes at room temperature. All suspensions used as inocula for tissue cultures contained 200 to 500 mcg. and units per milliliter of streptomycin and penicillin, respectively, and were stored in glass sealed ampules in the dry ice chest if not tested immediately.

Two types of roller tube cultures from tissues of either rhesus or cynomolgus monkeys were used primarily†: monolayer cultures[4] of trypsinized kidney, and plasma clot cultures of testicular tissue. Kidney cultures were maintained on medium 199 with 2 per cent inactivated horse serum. A mixture of equal volumes of 199 and commercial bovine amniotic fluid containing 2 per cent inactivated horse serum and 0.1 mg. per milliliter soybean trypsin inhibitor constituted the medium for testicular cultures. All culture media contained 100 mcg. and 100 units per milliliter of streptomycin and penicillin, respectively. Kidney cultures were changed to fresh medium at 3- to 5-day intervals, while testicular cultures were changed every 5 to 7 days. For the few experiments in which human kidney tissue‡ was employed, the roller cultures were prepared in the same manner as monkey kidney.

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‡The cooperation of Dr. Anthony Susan and staff of the Children's Hospital of Pittsburgh made available surgical specimens of tissue.

All serum samples from suspected exanthem patients were stored at −15° C. Many sera from other individuals which were screened for antibody had been stored at +5° C. For neutralization tests all sera, either undiluted or after 1:2 dilution with phosphate buffered saline, were inactivated at 56° C. for 30 minutes.

A fixed dilution of virus was added to twofold dilutions of serum so that the final inoculum of the mixture contained a calculated 10 to 100 tissue culture infective dose (TCID$_{50}$). Serum-virus mixtures were kept at +5° C. for 1 to 1½ hours, after which 0.1 ml. of the mixture was added to each of 2 roller tubes. Controls of virus plus buffer solution instead of serum were included with each test and in many of the experiments known positive serum controls also were included. Pools of infective tissue culture fluid used for neutralization tests were stored in ampules in dry ice and a titration was carried out with each test. The acute phase and the convalescent serum from a given patient were tested simultaneously. Inoculated cultures were generally changed to fresh media only once during an observation period of 10 to 12 days. Further details regarding neutralization tests are included in the text.

EXPERIMENTAL

Isolation of Agents From Clinical Specimens.—

From the community outbreak. Stool specimens from 11 clinically diagnosed cases of Boston exanthem disease were tested in cultures of either monkey kidney or testicular tissues. Fecal specimens were collected within 2 to 10 days after onset of illness. Serially transferable agents, which produced cytopathogenic effects indistinguishable from one another, were isolated from all 11 patients. Each isolate was passed at least once in both kidney and testicular cultures with the production of characteristic types of cellular destruction.

Either throat or mouth washings, collected within 3 days of onset of illness, were also available from 3 of the 11 patients whose stools were tested. Similar agents were recovered from throat washings of 2 of the cases (Mr. McG. and Mr. W.), but mouth washings from the third case (R. W.) were negative. A fourth patient whose throat washings were tested (Mrs. W.), but from whom no stool specimen was available, was negative. This patient had a characteristic clinical illness and was the only case of those tested in the community outbreak in whom isolation of a cytopathogenic agent was not achieved.

Recovery of virus from a stool specimen of a family contact, who also was reported to have had a characteristic illness with rash, constituted the twelfth isolation of an agent in association with the suburban community outbreak.

Isolations from subsequent sporadic cases. Stool specimens from 6 cases which occurred later in 4 widely separated areas of the city were tested in cultures of monkey kidney tissue. In each instance an agent was recovered which produced cytopathogenic changes similar to those isolated from the community outbreak. Data summarizing the origin of virus isolations from all the Boston exanthem cases in Pittsburgh are presented in Table I.

Relative susceptibility of several different tissues for primary isolation. Most specimens were first tested in roller cultures of monkey kidney, but all negative and most positive specimens were also tested in monkey testicular cultures. It soon became apparent that testicular cultures were more sensitive indicators of the cytopathogenic effects of these particular agents than were the kidney cultures.

TABLE I. VIRUS ISOLATIONS AND SERUM NEUTRALIZATION TESTS IN PITTSBURGH PATIENTS WITH BOSTON EXANTHEM DISEASE

PATIENT	SEX	AGE IN YEARS	VIRUS ISOLATION		SERUM NEUTRALIZATION TESTS*		
			MATERIAL TESTED	RESULT	DAY OF DISEASE	TITER VS. PITTSBURGH STRAIN	TITER VS. BOSTON STRAIN
Cases from Community Outbreak							
S. McG.	F	5/12	Feces	+	No specimens obtained		
D. McG.	M	4	Feces	+	6 27	4 16 to 32	2 16
R. McG.	M	6	Feces	+	6 27	4 16	4 to 8 32
Mr. McG.	M	29	Blood clot Throat wash. Feces	+ + +	2 23	<2 8	<2 4
R. Wel.	M	5	Blood clot Mouth wash. Feces	0 0 +	3 20	<2 8 to 16	<2 8
K. Wel.	M	6	Blood clot Feces	0 +	3 20	<2 8	<2 16
Mrs. Wel.	F	30	Blood clot Throat wash.	0 0	2 17	<2 32	<2 16
Mr. Wol.	M	30	Blood clot Throat wash. Feces	0 + +	2 23	– –	2 16
Mrs. Wol.	F	29	Feces	+	4 27	2 8	4 16
Mrs. B.	F	28	Blood clot Feces	0 +	4 21	2 32	<2 16
Mr. D.	M	35	Feces	+	3 18	<2 32	<2 16
T. F.	M	5	Feces	+	5 26	2 8	<2 4 to 8
L. L.	F	6	None	–	9 28	8 32	>8 >32
J. P.	F	2	Feces	+	No specimens obtained		
Sporadic Cases							
A. J.	F	2/12	Feces	+	No specimens obtained		
Mrs. J.	F	36	Feces	+	5 22	4 8	4 4
A. R.	F	7/12	Blood clot Feces	0 +	3 20	2 4	<2 8
E. N.	M	1	Blood clot Feces	0 +	4 18	<2 4 to 8	<2 8
E. W.	M	8/12	Blood clot Feces	0 +	5 No second specimen	<2	2
B. T.	M	2	Blood clot Feces	0 +	? ?	4 16	4 16

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*Done in tissue cultures, using 5 to 50 (TCID_{50}) of virus. Titers are reciprocal of serum dilution before virus was added. Pittsburgh strain was isolated from R. Wel. in 1954. Boston strain was isolated during 1951 outbreak in Boston, Mass.

Seven specimens, positive in kidney, were also positive in testicular cultures. However, 4 specimens negative in kidney were found positive when comparable inocula were tested in testicular cultures. In addition, several other specimens produced questionable effects on the first passage in monkey kidney and required a second passage for definite cytopathic changes. In either type of culture, first evidence of cytopathogenic changes was noted between the third to the eleventh day after inoculation.

The difference in susceptibility between monkey kidney and testis was also evident when some specimens were titrated for infectivity in both types of cultures. Ten per cent stool suspensions from several patients were found to contain detectable virus when diluted $10^{-3.0}$; in general, titers in monkey kidney were tenfold to a hundredfold lower than those in testicular cultures. Data on the comparative susceptibility of monkey kidney and testicular tissue for primary isolation are summarized in Table II.

TABLE II. SUSCEPTIBILITY OF MONKEY TESTICULAR VERSUS KIDNEY CULTURES TO AGENTS ISOLATED FROM EXANTHEM PATIENTS

PATIENT AND SPECIMEN	MONKEY KIDNEY CULTURES			MONKEY TESTIS CULTURES		
	INOCULUM		RESULT*	INOCULUM		RESULT
Mr. McG Blood Clot	0.25 ml to 5 tubes		0/5 Positive	0.4 ml to 4 tubes		4/4 Positive
Mr. McG Feces	0.1 ml to 4 tubes		0/4 Positive	0.1 ml to 3 tubes		2/3 Positive
Mr. McG Throat wash	0.4 ml to 5 tubes		1/5 Positive	0.5 ml to 4 tubes		4/4 Positive
K. Wel. Feces	0.1 ml at	10^0 10^{-1} 10^{-2}	3/3 Positive 0/3 Positive 0/3 Positive	0.1 ml at	10^{-1} 10^{-2} 10^{-3}	2/2 Positive 3/3 Positive 0/4 Positive
A. Roh. Feces	0.1 ml at	10^{-1} 10^{-2} 10^{-3}	1/3 Positive 0/3 Positive 0/3 Positive	0.1 ml at	10^{-1} 10^{-2} 10^{-3}	2/2 Positive 3/3 Positive 1/4 Positive

*Numerator represents the number of cultures showing degeneration; the denominator is the number of tubes inoculated.

Subsequent experiments using only slight modifications in nutrient media* with cultures of different types of human tissue have revealed the following comparisons of susceptibility to these agents: several different strains tested in trypsinized cultures of human kidney produce minimal or no obvious cytopathogenic changes, but further passage in these tissues may increase cytopathic effects. Human foreskin and embryonic skin muscle cultures are approximately equal in susceptibility, either upon primary isolation or after passage. Trypsinized human amnion cultures show variable degrees of cytopathic changes, but are less susceptible than human fibroblasts. Cultures of HeLa cells have shown no obvious changes up to 12 days after inoculation.

Attempts to isolate agents from the blood of patients. Frozen suspensions of clotted blood from 10 patients, bled on the second to fifth day of disease, were tested in roller cultures of monkey testis. Inocula per tube varied from 0.2 to 1.25 ml., and in many instances a further blind passage was carried out. An agent similar to those isolated from the feces was recovered from only one blood specimen (Mr. McG.). In this instance each of 4 tubes inoculated with 0.3 ml. produced characteristic changes within 5 to 7 days. It is of interest that the blood from the positive case was drawn when the patient had a fever of 104° F. while all negative bloods were from afebrile patients even though collected on the second or third day of illness.

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*The media consisted of 90 per cent bovine amniotic fluid, 5 per cent horse serum and 5 per cent beef embryo extract.

Tests for Neutralizing Antibody.—

General comments regarding neutralization tests in tissue culture: It has been observed previously[6] that neutralization of the exanthem agents by human convalescent serum was difficult to demonstrate unless minimal amounts of virus were used in the neutralization mixture. Even when 50 $TCID_{50}$ of virus are employed in a neutralization test, the phenomenon of virus "breakthrough," or progressive decrease in titer of a serum is seen with the passage of time.

The possibility was considered that changing the media of cultures during the test might contribute to the incomplete neutralization observed above. However, identical tests in which one set of cultures was changed and one set was kept with the original medium showed no striking difference in the results. Conditions of the test were further modified in an attempt to obtain more complete neutralization of virus by specific antibody. Incubation of the serum-virus mixture for one hour at 37° C. instead of at refrigerator temperature failed to enhance neutralization. The use of graded dilutions of virus with a constant serum dilution likewise appeared to offer no particular advantage.

In view of the above enumerated limitations, the only available test at present for in vitro neutralization has utilized the criterion of 2 to 3 days' delay in appearance of cytopathic changes afforded by convalescent as compared to acute phase or control serum when tested at the same time. Under these conditions, values of antibody titer are necessarily arbitrary. However, upon the basis of numerous tests, presence or absence of neutralizing antibody can be readily determined and reproducible estimates of titer have been possible.

Neutralizing antibody response in patients: Paired samples of acute and convalescent phase sera were available from a total of 16 cases with clinical evidence of Boston exanthem disease. Twelve of these patients represented cases from the community outbreak, and 4 were later scattered cases. Acute and convalescent sera from each case were tested simultaneously for their ability to neutralize 2 different strains of virus. One of the strains had been isolated from feces of an exanthem patient in Pittsburgh during 1954, the other strain originated from feces of an exanthem patient in Boston during the 1951 outbreak. Five to fifty $TCID_{50}$ of virus were used in the tests. Serologic results, along with other information concerning the patients, are presented in Table I.

The neutralizing antibody response in patients to a Pittsburgh strain as compared with a Boston strain showed close agreement. In most cases a fourfold or greater rise in antibody titer could be demonstrated to both strains, and only one patient failed to show significant development of neutralizing antibody to at least one of the strains. In this case (Mrs. J.) antibody was already present in the acute phase serum, but the onset of illness was so poorly defined that duration of disease could not be accurately determined

*Neutralizing antibody response in monkeys** The development of neutralizing antibody in either cynomolgus or rhesus monkeys could be demonstrated after primary intravenous or intracerebral inoculation followed by 2 intramuscular doses of infectious tissue culture fluid over a period of several months This was shown with 6 different strains representing isolates from exanthem patients in Pittsburgh as well as cases from the first outbreak in Boston Sera from 13 normal monkeys were negative for neutralizing antibody when tested at a 1 2 dilution Neutralization of heterologous strains with a given monkey antiserum has been demonstrated, but the extent of antigenic relationship is still under investigation

Failure to Associate Exanthem Agents With Other Illnesses—During the year preceding the outbreak of exanthem disease cases in Pittsburgh, stools from approximately 90 patients with suspected poliomyelitis from that same city were studied These were tested† in cultures of monkey kidney, and although a few unclassified cytopathogenic agents were isolated none resembled the agents described herein During the same season as the exanthem outbreak at least 30 fecal specimens from poliomyelitis suspects were likewise negative in monkey kidney culture for agents similar to those recovered from exanthem patients

Negative results for either enteric cytopathogenic viruses or specific neutralizing antibody responses were obtained in the following illnesses 3 patients with roseola infantum, 3 patients with German measles and 6 patients with fever and unclassified skin rashes Similar studies of 3 patients with vague minor illnesses in residents of the same community where cases of Boston exanthem disease occurred were also negative

Serum Survey for Neutralizing Antibody to Boston Exanthem Agents—

Results with gamma globulin One lot of human immune serum globulin, and one lot of gamma globulin from convalescent German measles blood, both of recent origin, were tested ‡ Neither of the lots had detectable neutralizing antibody to one of the exanthem strains at dilutions greater than 1 10 Six other lots of outdated gamma globulin were also tested, only one of the lots showed a detectable level of neutralizing antibody at 1 10, the lowest dilution tested However, these latter preparations may have decreased in potency with prolonged refrigerator storage

Results with sera from different age groups Serum samples collected in 1953 and 1954 from the Pittsburgh area from patients with poliomyelitis and other illnesses were tested for neutralizing antibody to a Boston strain of exanthem agent Each serum was inactivated and tested at a 1 2 dilution with 50 $TCID_{50}$ of virus in 2 roller cultures

The results are summarized in Table III Since there was *no* striking difference in incidence of neutralizing antibodies from sera collected in 1953 as compared to the 1954 specimens, the number tested in each age group represent both years of collection. Neutralizing antibody could be demonstrated in 5 per cent of the group less than 5 years old, 19 per cent of those 5 to 15 years old and in only 4 per cent of the group over 15 years of age. Some of the sera showed equivocal neutralization. This partial neutralization was found in only one or two specimens from either of the younger age groups, but 7 (15 per cent) of those over 15 years of age gave a doubtful result. Repeat tests with a number of the sera showed no change from the original results.

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*These experiments were done jointly with Dr L. James Lewis

†Many of these specimens were tested by Dr Gisela Ruckle in this laboratory

‡These lots of gamma globulin were kindly made available by Dr Johannes Ipsen Institute of Laboratories of the Massachusetts Department of Public Health

TABLE III. PRESENCE OF NEUTRALIZING ANTIBODY TO BOSTON EXANTHEM AGENT IN PITTSBURGH SERA, 1953-1954

	AGE GROUP		
	< 5 YEARS	5-15 YEARS	> 15 YEARS
Number tested	39	62	45
Number positive	2 (5.1%)	12 (19.4%)	2 (4.4%)
Number doubtful	1 (2.6%)	2 (3.2%)	7 (15.5%)

DISCUSSION

The viruses described were readily isolated from the feces of almost every Pittsburgh patient with the clinical picture of Boston exanthem disease. Throat washings, in contrast, appeared to be less suitable for recovery of these agents. Isolation from the blood was successful in only one of many attempts suggesting either that viremia occurs rarely, or that the blood specimens tested were obtained too late in the course of the illness.

The differences of in vitro sensitivity among various tissues must be kept in mind if isolation of these agents is attempted. Cultures of monkey kidney were adequate in most instances for primary isolation, although they were less susceptible than monkey testis. Human kidney cultures showed either no, or only equivocal, cytopathic effects. Cultures of human embryonic skin muscle or foreskin have shown approximately the same susceptibility as monkey testis.

Tests for neutralizing antibody in sera from Pittsburgh exanthem patients showed essentially the same results when tested with a Pittsburgh strain of virus as with a strain representing the earlier Boston outbreak. Thus, close antigenic relationship was demonstrated between agents recovered from patients with similar diseases that occurred several years apart in two widely separated cities.

The neutralization test with this group of viruses is still not entirely satisfactory. With the methods described, it is possible to obtain fairly reproducible evidence of neutralization. However, the factor of incomplete neutralization, or virus "breakthrough," necessitates arbitrary interpretation of the test, and constitutes a drawback in serologic work.

The results of the screening tests for neutralizing antibodies suggest that a moderate incidence of exposure to these agents had occurred during childhood. The low incidence of definitely positive sera and the relatively large number of doubtful results in the adult age group is more difficult to interpret. However, this may indicate a decline in levels of neutralizing antibody from infections acquired during childhood, as well as a low incidence of infection in adults. The results of tests for antibody in gamma globulin are consistent with the low incidence of definitely positive sera encountered in the screening of adults, and also agrees with the relatively low antibody titers found even in recently convalescent patients.

A high degree of association has been demonstrated between a recognizable clinical entity and the specific agents recoverable from such patients. This relationship of agents to disease was found not only in the setting of a localized outbreak, but also in scattered sporadic cases occurring in a large metropolitan area. The agents isolated from patients with the Boston exanthem disease could not be recovered from certain cases of other known or unclassified febrile exanthems, or from many patients with other illnesses which occurred in the same area at approximately the same time. A similar association of related or identical agents with the same type of illness has been reported previously.[6] These observations strongly suggest an etiologic relationship between this group of viruses and the illness referred to as Boston exanthem disease.

Further information, however, is needed to establish conclusively the etiologic role of these agents. The number of patients who have been studied may be insufficient to fully define the range of possible clinical manifestations. It is not yet known whether infection with these agents is invariably associated with a specific clinical illness. Finally, the existence of other related agents as yet unknown, cannot be excluded. For example, it has been found recently that certain enteric viruses recovered from several patients with aseptic meningitis that occurred during the first reported epidemic of Boston exanthem disease are related immunologically to the viruses described in this paper. The extent of immunologic relationship and comparison of other characteristics is under investigation.

SUMMARY

Study of a 1954 outbreak of Boston exanthem disease in Pittsburgh resulted in the isolation of characteristic agents cytopathic for tissue cultures from 18 patients. The viruses were recovered most readily from the feces, irregularly from throat washings, and once from the blood of patients. Cultures of monkey testicular tissue were found to be superior to those of monkey kidney for primary isolation. Neutralizing antibody responses in Pittsburgh patients indicated a close antigenic relationship of local strains to similar agents associated previously with the same type of disease in Boston. Results of screening tests of various age groups for neutralizing antibody are presented. The observations presented strengthen the likelihood that these viruses are etiologically related to the Boston exanthem disease.

We are indebted for advice to Dr. Jonas E. Salk in whose laboratory these studies were carried out.

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LABORATORY METHODS

THE COLLECTION OF LYMPH FROM INTACT PERIPHERAL CUTANEOUS LYMPHATICS OF MAN

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AS PART of a study of the role of the lymphatics in the formation of edema in congestive heart failure, a method was developed for the cannulation and collection of fluid from intact cutaneous lymphatics of the extremities of man. Whereas the chemical content of lymph from animals and from the thoracic duct of man has been studied extensively,[1, 2] there are relatively few data concerned with the electrolyte content and lymphodynamics of intact lymphatics in the extremities of man. The method presented is applicable to these as well as other studies of peripheral lymphatics and lymph under various circumstances without severe disturbance of the locale studied.

MATERIALS AND METHODS

The lymph channels are first visualized by the intradermal injection of 0.1 ml. sterile 11 per cent aqueous solution of Patent Blue V* with a 30 gage needle.[3-5] This quantity produces streamers intense enough for cannulation in 10 to 20 minutes. The longest and most intense streamers are most commonly seen on the forearm of normal subjects, whereas in edematous extremities there is rapid diffusion of the dye and distinct channels are sometimes difficult to discern.[5] The use of red fluoroscopic glasses facilitates the visualization of the blue stained lymphatics and differentiation from veins. All studies were performed after 30 minutes horizontal rest of the extremity at 76° F. and no attempts were made to induce or hasten streamer formation by exercise or application of heat to the part.[4]

Cannulation is performed counter to the current of flow with either a 23 gage needle from which the hub has been removed or a one inch 30 gage needle modified by removing the hub and soldering it into a ½ inch length of 23 gage stainless steel tubing (Figs. 1, 2, and 3). A suitable length of plastic tubing designed to fit snugly on a 23 gage needle† is attached as a collecting vessel with a U tube phlebomanometer[6] as an aspirator and indicator of the negative pressure applied. A three way stopcock permits recompression of the bulb, if necessary, without raising the pressure in the collecting system. If a syringe is used for aspiration, care must be taken not to exert a pressure so great as to collapse the small vessel or draw air into the plastic tubing through the needle puncture site or any of the junctions in the system. When this occurs a broken column of fluid is obtained, handicapping volume estimation, permitting evaporation from a greater surface area, and

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†B-D 442T

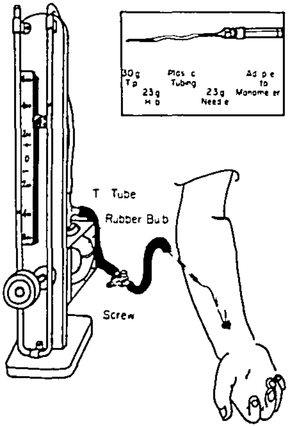

Fig 1.—The technique of cannulating a cutaneous lymphatic and collecting lymph. The phlebomanometer serves as a regulated aspirator. The 3 way stopcock in the line permits recompression of the bulb if the pressure falls due to leakage in the system. The insert shows the modified 30 gage needle attached to the collecting tubing and adaptor to aspirator. A 23 gage needle with the hub removed can be used as the cannula just as well.

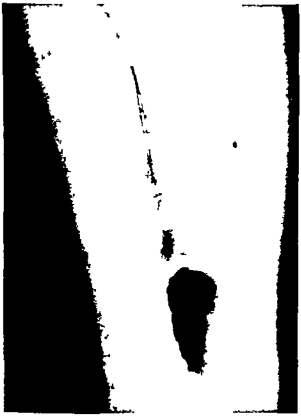

Fig 2

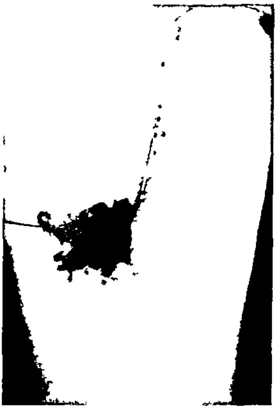

Fig 3

Fig 2.—Collection of lymph from a streamer in the arm of Subject 23D (CHF). This picture was taken 70 minutes after injection of the dye and 19 minutes after collection of lymph was begun. Stained lymph can be seen in the plastic tubing just above the cannula.

Fig 3.—Collection of lymph from the leg of Subject 24 (CHF) illustrating undesirable fragmentation of the column of fluid by air pulled into the collecting tubing during forced aspiration.

increasing the difficulty of expelling the fluid from the plastic tubing (Fig. 3). This is rarely encountered when a negative pressure of 200 to 500 mm of water is applied, but the time required to determine whether or not the vessel has been entered is longer. Since the rates of flow are small, 5 to 10 minutes should elapse before the investigator assumes he is not in the lymphatic and spoils a good cannulation by premature withdrawal of the needle.

If an unbroken column of fluid is collected in the plastic tubing, the quantity can be estimated periodically during the collection by measuring the length of the column. Measurements under controlled conditions showed the relationships of the weight of the fluid delivered to the length of a fluid column in the tubing to be 0.25161 mg per millimeter ± 0.00215 (Fig. 4). For precise measurement of the quantity of fluid delivered for chemical determinations, the tubing is weighed before and after the fluid is expelled into the desired container. Loss of fluid by evaporation from the open ends of the tubing during the interval before weighing may be reduced by drawing the fluid a short distance from the distal end and clamping. The rate of evaporation from tubing so clamped was measured at 76° F. and found to average 0.6 per cent per hour for a 24 hour period.

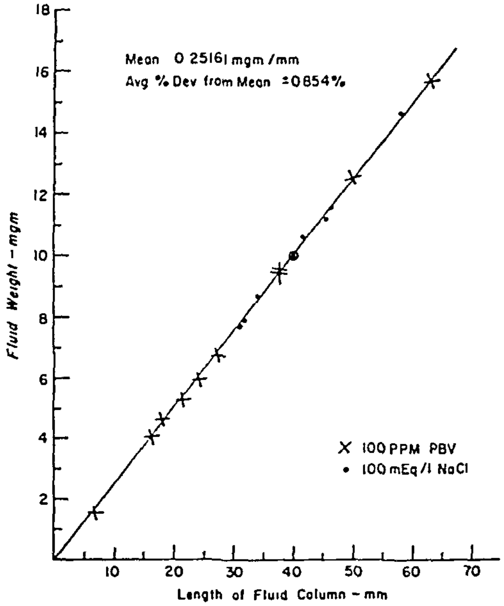

Fig. 4.—Relationship of the weight of fluid delivered from the plastic tubing to the length of the column of fluid in the tubing (B-D 442 T). Each millimeter of fluid in the tubing weighs 0.25161 mg. There were no detectable differences when PBV, sodium chloride or serum was used for calibration.

In the determination of the electrolyte content of the lymph, contamination of the small volume collected has been minimized by expelling the lymph from the plastic tubing into siliconed 4 ml. weighing bottles which have been rinsed at least 10 times with triple distilled water and dried in room air while covered to protect from dust particles. Triple distilled water is also used as the diluent. Concentration of sodium in such samples has been determined with a Beckman flame photometer and concentration of chloride by potentiometric titration with silver nitrate of a 1 ml. aliquot of that diluted for sodium determination, evaporated to dryness and redissolved in 0.2 ml. 50 per cent acetic acid.[7, 8]

The concentration of radioactive tracers has been determined by delivered onto disks for counting.[9] Pressures within lymphatics have been measured by the Burch technique for venous and tissue pressures.[10]

RESULTS

The number of experiments, subjects studied, and cannulations is presented in Table I. Lymphatics were entered in 62.6 per cent of the attempted cannulations as evidenced by the appearance of the blue tinged lymph in the tubing or at the site of the puncture after withdrawing the needle. In 36 instances enough lymph was obtained for gravimetric quantification. In 15 (13 per cent) of the cannulations blood was obtained from vessels which contained dye and which were indistinguishable by inspection from streamers which yielded lymph.

TABLE I. TABULATION OF COLLECTION OF LYMPH

	CONTROLS		CHF		OBSTRUCTIVE		MISC		TOTALS		
	ARM	LEG	ARM	LEG	ARM	LEG	ARM	LEG	ARM	LEG	TOTAL
Number subjects	11	4	3*	5*	0	3	1†	3‡	15*	15*	28*
Number experiments	13	4	3	5	0	4	1	3	17	16	33
Attempted lymphatic cannulations	43	20	9	21	0	11	2	9	54	61	115
Lymphatics entered	27	9	6	11	0	9	2	8	35	37	72
Quantity sufficient for measurement	8	5	5	7	0	5	1	5	14	22	36
Blood obtained from streamer	3	3	2	5	0	1	0	1	5	10	15

CHF = Congestive heart failure.

*Two subjects were studied in the leg on one occasion and in the arm on repeated study.

†Chronic glomerulonephritis, no edema.

‡One edema, undiagnosed etiology; 1 cirrhosis, edema; 1 myocardial infarction, no failure, no edema.

The rates of collection of lymph according to the type of subject and size of the cannula are presented in Fig. 5. This figure and the time courses of the cumulative collection of lymph from some of the subjects shown in Fig. 6 illustrate that the rates of collection were greatest and volumes collected largest from the edematous legs of subjects with congestive heart failure when a 23 gage needle was used. The quantities of lymph collected from all subjects varied with the duration and rate of collection and ranged from 0.15 mg. to 19.0 mg.

Twenty-nine samples collected were analyzed for sodium. Values for the 18 samples analyzed after problems of obvious contamination were alleviated are presented in Table II, A. The concentrations of chloride determined by the micropotentiometric titration are included in this table. In 9 studies edema fluid was obtained directly without cannulating the lymphatics. The volumes collected in these instances were of sufficient magnitude to permit standard methods of determination of electrolyte or protein content (Table II, B).

The concentration of dye in the lymph of 5 subjects ranged from 0.005 to 6.7 per cent of the concentration injected. In three studies after radioiodinated serum albumin* was injected at the same site as the dye, concentration of I^{131}

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*Risa, Abbott Laboratories, North Chicago, Ill.

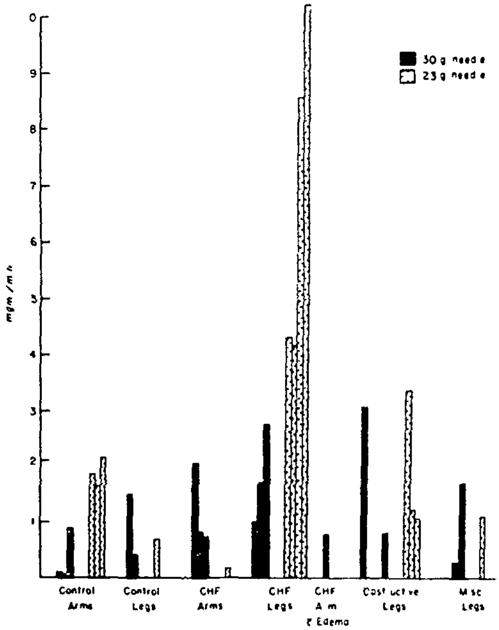

Fig. 5 —The rates of collection of lymph from various subjects with either a 23 or 30 gage needle The greatest rates of collection were apparent when a 23 gage needle was used to collect from subjects with congestive heart failure

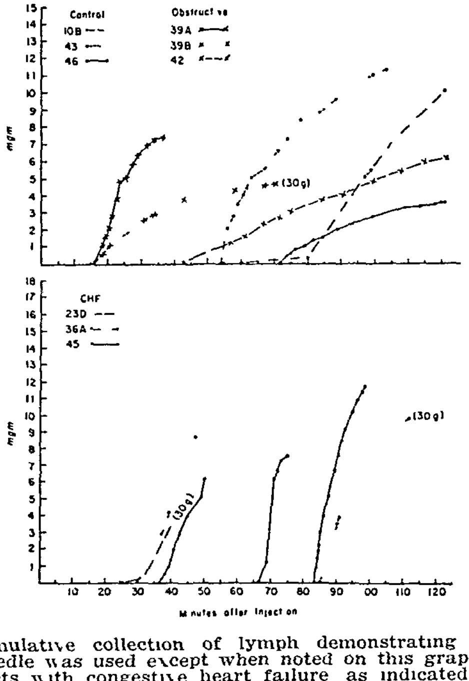

Fig 6 —The cumulative collection of lymph demonstrating the time course of collection A 23 gage needle was used except when noted on this graph The collection appears to be fastest in subjects with congestive heart failure as indicated by the steeper slopes of the lower set of curves The weight in milligrams has been calculated from the column length using the calibration factor 0 25161 mg per millimeter

in the lymph varied from 0.005 per cent to 2.9 per cent of the concentration of the injected counts. Such variations resulted from differences in times of collection after injection and in distances from the injection site. These factors must be controlled before such data can be used to evaluate physiologic influences, such as variations produced by differences in uptake by the lymphatics or variations in rates of flow within the vessels. In one subject the concentration of dye in blood obtained from what appeared to be lymphatics was 2 per cent of the injected concentration of dye.

TABLE II, A. ELECTROLYTE CONTENT OF LYMPH

SUBJECT NO.	DIAGNOSIS	SITE	EDEMA	SODIUM (MEQ./L.)		CHLORIDE (MEQ./L.)		PROTEIN (GM. %)	
				SERUM	LYMPH	SERUM	LYMPH	SERUM	EDEMA
32	Control	Arm	0	143.5	148.2				
33	Control	Arm	0	140.5	148.2				
43	Control	Arm	0	140.0	138.8	106.0	107.3		
46	Control	Leg	0	143.5	154.5				
48	Control	Arm	0	140.5	138.8				
11.3	Control	Leg	0	141.7	138.6				
23D	CHF	Arm	4+ in leg	131.0 131.0	127.9 132.9				
36	CHF	Leg	3+	141.2	137.5				
				141.2	145.2	111.0	100.1		
				141.2	142.4				
45	CHF	Leg	3+	133.0	128.1				
39A (6/18)	Obstructive	Leg	0	139.5	150.3				
				139.5	135.2				
39B (6/25)	Obstructive	Leg	3+	136.7	137.3				
42	Obstructive	Leg	4+	143.2	153.7				
44	Cirrhosis	Leg	3+	126.5	127.6	95.0	101.9		
				126.5	129.2				

TABLE II, B. ELECTROLYTE CONTENT OF EDEMA FLUID

SUBJECT NO.	DIAGNOSIS	SITE	EDEMA	SODIUM SERUM	SODIUM LYMPH	CHLORIDE SERUM	CHLORIDE LYMPH	PROTEIN SERUM	PROTEIN EDEMA
23A (10/4)	CHF	Leg	4+	134.0	137.8	97.5	102.5		
				134.0	142.5	97.5	105.6		
23B (10/18)	CHF	Leg	4+	125.0	129.0	100.6	109.7		
23C (11/14)	CHF	Leg	4+	129.2	131.2	88.3	95.9		
23D (12/13)	CHF	Leg	4+	131.0	131.4				
				131.0	131.0	91.1	99.6	5.75	0.54
19B	Obstructive	Arm	4+	144.0	124.5	102.8	107.6		*
11.1A (1/29)	Renal cancer	Leg	4+	131.3	131.3	86.2	95.2		
11.1B (1/31)	Renal cancer	Leg	4+	133.9	133.0	90.0	95.8	3.82 A 2.35 G 1.47	0.708 A 0.576 G 0.132

*Quantity insufficient for demonstration but was thick, viscous and yellow fluid.

On two occasions pressure within the lymphatics was determined with a phlebomanometer.[10] Because of slow flow and the small needles used, lags in the determination permitted only an estimate of the pressure within limits of 20 to 30 mm. of water in a normal subject and 50 to 75 mm. of water in a subject with obstructive lymphedema and venous disease. More accurate methods of determination are being adapted for use.

DISCUSSION

Although Patent Blue V was used for the visualization of the lymphatics in these studies, other dyes can be employed. Direct Sky Blue,* which has also been utilized satisfactorily in this laboratory, or other methods of visualization of the lymphatics, such as the use of fluorescent substances and ultraviolet light,[11] may be preferred by individual investigators. Although most of the blue streamers which developed after the injection of Patent Blue V were lymphatics, some proved to be blood vessels as demonstrated by the collection of blood containing dye and by serial photographs showing intensification of the color of small veins as dye entered them.[12] It is unlikely that this was due to the direct injection of venous channels, since from 5 to over 20 minutes were required for these vessels to become intensely blue or to develop the appearance of lymphatic streamers. These observations indicate that veins as well as lymphatics have been included in studies demonstrating "lymph channels" and measuring the rates of spread of dye.[3-5, 11] These studies also confirm the suggestion of Kinmonth that Patent Blue V is taken up by venous capillaries.[13]

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*Wyeth Inc., Philadelphia, Pa.

The quantity of data thus far collected by this technique is not sufficient for interpretative evaluation of the variations observed. There are some observations, however, deserving comment. In Table II, A it may be noted that some of the values for the concentration of sodium in lymph were higher than those in serum. Although a "control" serum was run with each sample of lymph there was no assurance against minute undetectable contamination of individual beakers used for the dilutions, which would yield results higher than the true concentration. Collection of more than one sample from the same individual as control of this was possible in some subjects (Table II, A). This type of error did not exist in the determination of the concentration of sodium in the serum or edema fluid since relatively large volumes of sample were available.

The rates of collection of lymph illustrated in Figs. 5 and 6 cannot be interpreted as indicating rates of lymph flow within the lymphatics since these studies were not designed to control all of the factors known to influence these rates of collection. The slightly faster rates of collection from subjects with edema, however, suggest that the size of the "lymph pool" or elevation of tissue pressure and pressure within the lymphatics plays a part in the facilitation of collection of lymph from edematous extremities regardless of flow within the vessels themselves.[5] Further investigation is designed to study the influence of these factors upon the rate of collection and to study rates of flow within the lymph vessels.

SUMMARY

A method for the cannulation and collection of fluid from the cutaneous lymphatics of the extremities of man after visualization of the lymphatics by the intradermal injection of a vital dye has been presented. In 33 experiments 115 lymphatic cannulations have been attempted. The lymphatics were entered 72 times, in one-half of which the quantity of lymph collected was sufficient for measurement. Blood was obtained on 15 occasions from streamers which appeared to be lymphatics, indicating that some studies of visualized lymphatics have probably included small venous channels as well. Since only a small

amount of data concerned with the electrolyte content of lymph has been collected, no attempt has been made to evaluate differences between subjects It has been pointed out that due to the small volumes of fluid and hazards of contamination the possibility of errors in these determinations exists Concentrations of electrolytes determined by standard techniques on larger volumes of edema fluid are presented for comparison

When a small volume of fluid is not a limiting factor, the collection of lymph by this technique has potential usefulness in the study of cytologic content, transport of crystalloids, colloids or tracer substances, antibody responses, or other processes in conditions not necessarily related to edema

The technical assistance of Miss Patricia J Grubbs and Mrs Mary J Smith is acknowledged

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THE DETERMINATION OF SERUM ALBUMIN AND GLOBULIN BY A NEW METHOD

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INTRODUCTION

RECENTLY two of us[1] showed that serum albumin, after precipitation with trichloroacetic acid (TCA), is soluble in alcohol. Moreover, albumin with similar physical properties to the native material can be recovered from the alcoholic TCA solution. The globulins, on the other hand, are completely denatured and are quite insoluble in alcoholic TCA. Later it came to our attention that the same type of observations had been made previously by Delaville and co-workers[2, 3] and Levine.[4] More recently this phenomenon has been investigated extensively by Schwert[5] who showed that the product obtained by solubilization in alcoholic TCA retains the same characteristics as albumin with respect to electrophoresis, sedimentation, crystallization, solubility, and thermal stability. We have made similar observations with respect to the first two of these characteristics, some of which we wish to report in this communication.

These observations make it possible to design a very simple method for the estimation of serum albumin and globulin, a method which can be readily adapted to a micro scale without loss of accuracy. This is made possible by applying the elegant method of Lowry and co-workers[6] to the measurement of the protein and the albumin solubility in TCA to separate serum albumin from the globulins.

MATERIALS AND METHODS

Reagents.—

Trichloroacetic acid: 15 per cent (micromethod).

Trichloroacetic acid: 1 per cent in 96 per cent alcohol (macromethod).

Sodium carbonate: 2 per cent in 0.1 N NaOH.

Copper sulfate: 0.5 per cent $CuSO_4.5H_2O$ in 1 per cent potassium (or sodium) tartrate.

Alkaline copper reagent: Mix 50 ml. of sodium carbonate reagent and 1 ml. of copper reagent. Make fresh each day.

Phenol reagent: (Folin-Ciocalteau phenol reagent.[7]) Place 100 Gm. sodium tungstate dihydrate, 25 Gm. sodium molybdate dihydrate, 700 ml. water, 50 ml. 85 per cent phosphoric acid, and 100 ml. conc. HCl in a 2 liter flask with a glass joint and equipped with a reflux condenser. Reflux gently for 10 hours, then add 100 Gm. lithium sulfate, 50 ml. H_2O, and a few drops of bromine. Boil for 15 minutes under the hood to remove the excess halogen, cool, and filter as necessary. Titrate a portion of the reagent with standard base using phenolphthalein indicator and, on the basis of the titer found, dilute the total volume so as to obtain a reagent 1 N in acid. The reagent should have a clear yellow color. Store in a glass stoppered bottle under dust-free conditions.

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Albumin standard 1 per cent crystalline bovine serum albumin* in water

Ethanol 96 per cent

Procedure —

Macromethod Total protein 1 Measure 0 5 ml of serum into a 10 ml volumetric flask and dilute to volume 2 Determine the chromogenic value on 10 lambda samples as described below

Macromethod Albumin 1 Dilute 0 5 ml of serum to 10 ml with the alcoholic TCA solution Mix well by inversion 2 Centrifuge down the globulins 3 Determine the chromogenic value on 10 lambda samples of the supernatant

Micromethod Total protein 1 Wash 10 lambda of serum with a micro pipette into approximately 100 lambda of water contained in a 10 ml volumetric flask Dilute to volume 2 Determine the chromogenic value on 1 ml aliquots of the diluted serum

Micromethod Albumin 1 Wash 10 lambda of serum into 100 lambda of water 2 Precipitate with 200 lambda 15 per cent TCA 3 Make to 1 ml with alcohol (96 per cent) Shake well 4 Centrifuge down the globulins 5 Determine the chromogenic value on 100 lambda samples

Development of color 1 Make the aliquot of the protein containing solution up to 2 ml 2 Add 5 ml of the carbonate copper reagent 3 Mix and allow to stand 10 minutes 4 Add 0 5 ml of the diluted Folin Ciocalteu reagent rapidly and mix well 5 Make a reagent blank as above but omit the protein 6 After 30 minutes read the optical density at 780 mμ in a spectrophotometer (or in the Klett with the red filter) 7 Subtract the value of the reagent blank from the unknown 8 Compare with the standard albumin or use a standard curve to determine the protein

TABLE I EFFECT OF TCA AND ALCOHOL ON PROTEIN IN SUPERNATANT*

	ALCOHOL (PER CENT)	
	70	90
TCA NORMALITY	OPTICAL DENSITY	
0 312	0 094	0 096
0 390	0 0845	0 083
0 468	0 072	0 071
0 546	0 072	0 072
0 624	0 072	0 072
0 702	0 070	0 071
0 780	0 0715	0 072
1 0	0 071	0 071

*To 100 lambda of serum diluted 1 to 10 was added 200 lambda of TCA of the strength indicated in the first column The volume was then made up to 1 ml Color was generated as described in the text

TABLE II RECOVERY OF ALBUMIN ADDED TO SERUM

	MIXTURE		A/G RATIO	
NO	ALBUMIN* (ML)	SERUM (ML)	CALCULATED	FOUND
1	0 0	0 5		1 00
2	0 25	0 5	1 56	1 55
3	0 50	0 5	2 11	2 05
4	0 75	0 5	2 66	2 62
5	1 00	0 5	3 22	3 30
6	0 0	0 5		1 01

* Albumin 4 0 per cent.

RESULTS

When the amount of TCA or alcohol used in the method is varied over quite a wide range, there is little effect on the amount of soluble protein This is shown by the data given in Table I Rather similar results were reported by French workers[2, 3]

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*Pentex Kankakee Ill.

DISCUSSION

It is quite evident that the method described for the determination of serum albumin is considerably simpler than any of those used at the present time in clinical work, and, since it can readily be carried out on a micro scale, it possesses considerable merit. Moreover, the method requires a minimum of reagents, only one of which is at all difficult to prepare and that one is available commercially (Folin-Ciocalteu reagent).

The color reaction used in this method is considerably more sensitive than the biuret reaction, and equivalent amounts to color are developed by all the serum proteins examined. A linear relationship exists between color developed and protein concentrations over a range of at least 25 to 125 μg of protein.

SUMMARY

A new micro method is described for the determination of serum albumin and globulin which depends on the solubility of the albumin in alcoholic trichloroacetic acid. The reagent renders the globulins insoluble by denaturation. Total protein and albumin in solution are determined by the use of the colorimetric method of Lowry and co-workers.

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A FLUOROMETRIC METHOD FOR THE ESTIMATION OF TYROSINE IN PLASMA AND TISSUES

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THE reaction between 1 nitroso 2 naphthol and tyrosine, under suitable conditions, yields a yellow product which has been utilized for the colorimetric estimation of tyrosine in biologic material It has now been found that the nitrosonaphthol derivative of tyrosine fluoresces Measurement of the fluorescence of this derivative endows the procedure of Udenfriend and Cooper[1] with greater sensitivity and specificity than was obtained in their original colorimetric method

In this report, the fluorometric method is used to determine the amount of tyrosine in plasma from fasting patients with different disease states, as compared to the amount of the amino acid found in the plasma of normal fasting controls A few of the patients selected had diseases in which it was thought that an alteration in tyrosine metabolism might occur The survey is not intended as a critical analysis of tyrosine plasma levels, but rather to demonstrate the method and its application

MATERIAL AND METHODS

Reagents—1 nitroso 2 naphthol 0 1 per cent 1 nitroso 2 naphthol (practical grade) in 95 per cent alcohol

Nitric acid reagent 24 5 ml of 1 5 nitric acid is mixed with 0 5 ml of 2 5 per cent $NaNO_2$

Procedure—The chemical method is the same as that previously reported (1) One milliliter of plasma is diluted to 4 ml with water, and 1 ml of 30 per cent trichloro acetic acid is added After ten minutes, the mixture is centrifuged To 2 ml of the deproteinized plasma, in a glass stoppered centrifuge tube, are added 1 ml of the nitroso naphthol reagent, and 1 ml of the nitric acid reagent The tube is stoppered, shaken, and placed in a water bath at 55° C for thirty minutes After cooling, 10 ml of ethylene dichloride is added, and the tube is shaken to extract the unchanged nitrosonaphthol reagent After the tube is centrifuged, the supernatant aqueous layer is transferred to a cuvette and read in the spectrophotofluorometer The fluorescence of the tyrosine derivative, resulting from its activation at 460 mμ, is measured at 570 mμ

Plasma was obtained from the blood of patients and of normal individuals who had fasted overnight but had not had any other dietary restrictions Heparin was added as an anticoagulant Duplicate plasma samples and a tyrosine recovery sample were run through the entire procedure with tyrosine standards and a reagent blank

The Aminco Bowman spectrophotofluorometer was used for measurement of fluorescence and for recording of activation and fluorescence spectra

RESULTS

In Table I are shown the actual readings obtained on the spectrophotofluorometer at the fluorescence peak of the tyrosine nitrosonaphthol derivative Values are shown for tyrosine standards, plasma, and for samples of

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TABLE I. DIRECT READINGS TAKEN FROM THE SPECTROFLUOROMETER FOR THE NITROSO-NAPHTHOL DERIVATIVE OF TYROSINE IN A PLASMA SAMPLE, STANDARD TYROSINE, AND TYROSINE ADDED TO THE PLASMA

SAMPLE	FLUOROMETER READING		TYROSINE RECOVERY* (PER CENT)
	DUPLICATES	AVERAGE	
Plasma†	51		
	51	51	---
Plasma + 5 γ tyrosine	68		
	70	69	100
Plasma + 10 γ tyrosine	86		
	84	85	98
Plasma + 15 γ tyrosine	96		
	98	97	91
5 γ tyrosine	18		
	18	18	---
10 γ tyrosine	35		
	37	36	---
15 γ tyrosine	56		
	56	56	---

*This is calculated on the basis of the expected reading, obtained by adding together the individual readings for plasma and the tyrosine standard.

†One milliliter of plasma was taken for assay.

TABLE II. FASTING PLASMA TYROSINE LEVELS IN NORMAL PATIENTS AND IN PATIENTS WITH VARIOUS DISORDERS

DIAGNOSIS	PATIENT NUMBER	TYROSINE (γ/ML. OF PLASMA)
Normal adult	1	12.1
	2	12.4
	3	11.6
	4	11.9
	5	10.3
	6	12.9
	7	13.0
Essential hypertension	8	11.9
	9	14.8
	10	9.9
Hypertension, congestive heart failure, mild diabetes	11	12.2
Hypertension, obesity	12	13.1
Arteriosclerotic heart disease	13	15.0
	14	15.2
Rheumatoid arthritis	15	11.2
	16	11.6
	17	11.0
	18	8.3
	19	13.1
	20	10.1
	21	9.0
Rheumatoid spondylitis	22	8.3
	23	13.1
Lupus erythematosus	24	9.9
Pheochromocytoma	25	8.0
		8.4*
		12.3†
Malignant carcinoid	26	9.7
	27	14.7
Hyperthyroidism	28	14.2
		13.2*
Malignant melanoma	29	10.3
	30	6.8
		9.5*

*Repeat analysis on a sample obtained at a later date

†Repeat analysis on a sample obtained after removal of the tumor.

the same plasma to which tyrosine had been added The recovery of the apparent tyrosine in plasma plus that added ranged from 91 to 100 per cent when compared to the total amount expected Recoveries of 91 per cent or better, calculated in the same way, were also obtained with all the plasma samples used in the studies shown in Table II

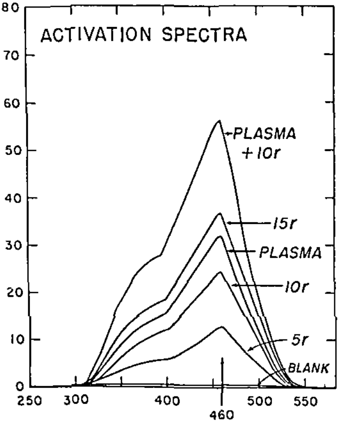

Fig 1

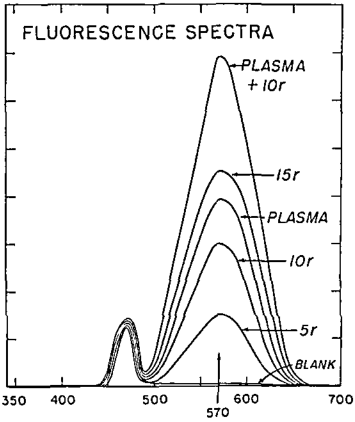

Fig 2

Fig 1 —Activation spectra for the nitrosonaphthol derivative of tyrosine from (1) standard solutions (2) plasma alone and (3) plasma plus 10 γ of tyrosine standard

Fig 2 —Fluorescence spectra for the nitrosonaphthol derivative of tyrosine from the identical samples used for Fig 1

In Fig 1 are shown the activation spectra of the tyrosine-nitrosonaphthol derivative from standard tyrosine solutions, from plasma alone, and from the same plasma to which 10 γ of tyrosine standard had been added Fig 2 shows the fluorescence spectra for the samples used in Fig 1 It is apparent that the nitrosonaphthol derivative from plasma has fluorescence characteristics identical with authentic tyrosine, being activated at 460 mμ and fluorescing at 570 mμ

Fasting plasma tyrosine levels in patients with specific diseases and in seven normal controls are shown in Table II Levels in the seven normal individuals ranged from 10 to 13 γ per milliliter of plasma A few of the patients exhibited levels above or below this range

DISCUSSION

Fluorometric methods of analysis are of value because of their great sensitivity as compared to other methods of assay of biologic materials Using the recently developed spectrophotofluorometer,[2] the sensitivity of the nitrosonaphthol method for tyrosine, as described here, is greatly increased in comparison to its colorimetric assay. For example, to read the samples in the plasma filtrates (10 to 15 γ) the spectrophotofluorometer was employed at a sensitivity setting which is actually a fraction of what could be utilized. It has been found that 1 γ or less of tyrosine can be measured by the procedure outlined in this method. In comparison, for a sample containing 10 to 15 γ of tyrosine, the optical density obtained in the Beckman spectrophotometer is only about 0.050, near the limits of its useful range. Moreover, the specificity of the fluorometric method is demonstrated by the similarity of the activation and fluorescence spectra of the nitrosonaphthol derivatives obtained with plasma filtrates and with authentic tyrosine. In contrast, the absorption spectrum of the nitrosonaphthol chromophore from plasma differs considerably from that of the standard tyrosine derivative.[1] The colorimetric method, as applied to plasma, is not specific although it has been shown to be more specific than other chemical methods for tyrosine assay.

The fluorometric method does not distinguish between tyramine and tyrosine; nor does the colorimetric assay. However, the amount of tyramine normally present in tissues is negligible, if present at all, and none is found in plasma.

Although some of the values for plasma from patients with specific diseases were above or below the normal range (10 to 13 γ per milliliter), an insufficient number of determinations was done to make any conclusions from the data. The values for tyrosine in human plasma found here agree well with those previously reported using a microbiologic method.[3]

The method can probably be applied to other biologic materials in addition to plasma. It should be particularly useful in studies where great sensitivity is required.

SUMMARY

A fluorometric method for tyrosine assay has been described. The specificity and sensitivity of the method are discussed, and its application to the determination of tyrosine levels in human plasma has been shown.

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ESTIMATION OF URINARY CATECHOL AMINES BY MEANS OF A STRIP OF RABBIT AORTA AS AN AID IN THE DIAGNOSIS OF PHEOCHROMOCYTOMA

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WITH THE TECHNICAL ASSISTANCE OF R. M. SANDERS

AN INCREASED urinary output of epinephrine and norepinephrine in pheochromocytoma has been verified both by bio-assay[1-3] and by chemical methods.[4] We have found, as have Furchgott and Bhadrakom,[5] that spirally cut strips of thoracic aorta of rabbits are admirably suited for the qualitative and quantitative assay of epinephrine and norepinephrine. Consequently, when asked to perform an assay for catechol amines on urine from a patient suspected of having a pheochromocytoma, urine was tested on the aortic strip. When 0.2 ml. of unfractionated urine was added to the muscle chamber, a maximal contraction occurred An equivalent amount of urine from a normotensive individual caused only an insignificant shortening From this response, it appeared that this technique might be useful for screening patients with hypertension for the possible presence of a pheochromocytoma. In this report the experience with this technique is presented.

MATERIALS AND METHODS

Spirally cut strips of rabbit thoracic aorta are prepared as described by Furchgott and Bhadrakom.[5] Rabbits weighing 4 to 6 pounds are killed by a blow on the head, and bled by severing the blood vessels in the neck The descending thoracic aorta is removed and placed in a Petri dish containing Krebs bicarbonate solution[6] at room temperature Excess fat and connective tissue are trimmed off The whole length of aorta is then cut along a close spiral. Strips of approximately 40 to 45 mm. in length (unstretched) are used for the assay. The strips are mounted in a muscle chamber of 20 ml. capacity with the aid of stainless steel hooks. The muscle chamber assembly is illustrated in Fig. 1. The bath is maintained at 37 5° C. The strip is bathed with Krebs bicarbonate solution containing 0 2 per cent glucose Through this solution, both in the muscle chamber and reservoir bottle, 95 per cent O_2 and 5 per cent CO_2 is bubbled to maintain the pH of 7 4 The isotonic lever is adjusted to give a ninefold amplification and counterweighted to exert 4 Gm. tension on the strips. The contraction is recorded on "Waxer" paper (Gorrell and Gorrell) by means of a heated stylus attached to the lever. A small vibrating motor is clamped to the rod holding the lever to eliminate any friction of the stylus on the paper

Collection and Preservation of Urine.—Immediately after the urine has been voided, it is adjusted to pH 3.0 by the addition of HCl to prevent inactivation of the catechol amines. Von Euler[7] has found that even after 15 days at room temperature at pH 1 to 3 catechol amines in urine are not appreciably inactivated We have found that when kept in a frozen state, catechol amines in urine at pH below 3 maintain activity for over a year.

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Since it is sometimes difficult to obtain 24 hour urine specimens in an open ward or outpatient department, the small amount of urine needed for assay with the aortic strip method makes it possible to collect urine over a shorter period. Most of the assays reported in this paper have been done on urine collected over a 2 hour period If the samples are not assayed at once, an aliquot is stored in a frozen state.

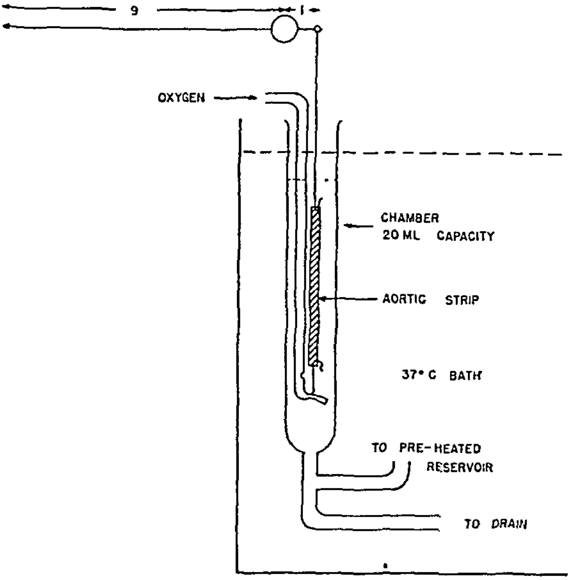

Fig 1—Muscle chamber assembly for aortic strip

Assay Procedure.—The strip is not used for assays until maximal sensitivity to norepinephrine or epinephrine is attained. This usually occurs after 2 or 3 additions of 0.05 to 0 1 μg of norepinephrine to the chamber. With these doses, contractions of 21 to 44 mm are obtained. The time required for the strip to reach maximal responsiveness is about 1½ hours.

In order to compensate for differences in urine volume, an amount of unfractionated urine equivalent to that excreted in 0 1 to 0 2 minute is added directly to the muscle chamber from a tuberculin syringe fitted with a needle long enough to dip below the surface of the solution in the chamber. The plunger is moved back and forth 3 times to insure proper mixing.

Test substances are washed out of the chamber within 1 to 2 minutes after maximal contraction is attained.

RESULTS

Qualitative Assay.—In Fig. 2 is shown an example of an assay of urine for catechol amines. Unfractionated urine equivalent to that excreted in 0.1 minute was added to the muscle chamber. The specimen from a patient suspected of having pheochromocytoma caused a strong contraction; whereas an equivalent amount of urine from a normotensive subject or from a patient with essential hypertension produced no shortening of the muscle strip. The specificity of the test was shown by the addition of 0.1 mg. Regitine (phentolamine methanesulfonate) to the muscle chamber and the retesting of the urine from the patient suspected of having pheochromocytoma. In the presence of Regitine, this urine was inactive. The strip was also unresponsive to *norepinephrine.*

Since the constrictor activity of angiotonin is not abolished by adrenolytic agents, angiotonin was added to the muscle chamber in order to test the viability of the strip. The resulting contraction indicated that Regitine did not have an injurious effect.

Since normal urine contains hydroxytyramine in addition to epinephrine and norepinephrine (von Euler, Hamberg and Hellner[8]), the possibility that this catechol amine might influence the assay had to be considered. Hydroxytyramine behaves like epinephrine and norepinephrine in two respects. First, a factor in urine enhances the ability of each of these agents to cause a contraction of the strip; second, Regitine also inactivates it. However, hydroxytyramine is only about one fiftieth as active as norepinephrine on the rabbit aorta strip and should have little effect on the assay.

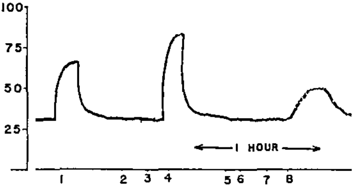

Fig. 2.—Assay of unfractionated urines for catechol amines on strip of rabbit aorta. 1, 0.5 mg. Versene + 0.15 µg norepinephrine. 2, normal urine equivalent to 0.1 minute. 3, urine from a patient with essential hypertension
toma urine equivalent to 0.1 minute. 5, Regiti
equivalent to 0.1 minute. 7, 0.5 mg. EDTA + 0.15
Ordinate, record of contraction in millimeters. Le

Additional evidence that hydroxytyramine does not interfere with the assay was obtained by the use of Marsilid (iproniazid*), an amine oxidase inhibitor. This agent markedly potentiates the contraction produced by both tyramine and hydroxytyramine. It has only a slight effect on epinephrine or norepinephrine on the strip. The addition of Marsilid to the muscle chamber has not enhanced the contraction produced by either normal urine or urine from patients with pheochromocytoma so far tested.

Weil-Malherbe[9] recently described a patient with pheochromocytoma in which the urinary excretion of hydroxytyramine was increased as much as or more than the excretion of noradrenaline. The addition of Marsilid to the bath would be useful in detecting such cases.

Further indications of the specificity of the test are demonstrated in Fig. 3, in which a urine from a patient with pheochromocytoma was assayed before and after the removal of the tumor. The preoperative urine specimen caused a strong contraction; the postoperative sample gave the same response as a urine from a normotensive subject. Identical results were obtained with urines collected before and after operation from 4 other patients.

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*I wish to thank Dr. Elmer L. Severinghaus of Hoffmann-La Roche Inc. for kindly supplying the Marsilid (iproniazid) used in this study.

Quantitative Assay of Pheochromocytoma Urine.—For screening hypertensive patients for the possible presence of a pheochromocytoma, a quantitative assay is not in most instances necessary. The difference between the contraction produced by urine from patients with essential hypertension and proved cases of pheochromocytoma that have been assayed in this laboratory has been obvious. The amount of urine equivalent to 0.1 to 0.2 minute of the former group of patients causes only an insignificant contraction, whereas with the latter group, an amount of urine equivalent to 0.02 to 0.1 minute has caused a strong contraction. However, between the two extremes are a group of patients whose urine causes a shortening of the muscle strip that is definitely more than that produced by average normal urine but not as much as that from proved cases of pheochromocytoma that we had examined early in our studies. In order to make a better interpretation of the results of the assay of such urines, a quantitative assay has been done on all urines that cause a definitely greater contraction than normal urine, as well as urines from a group of normotensive subjects.

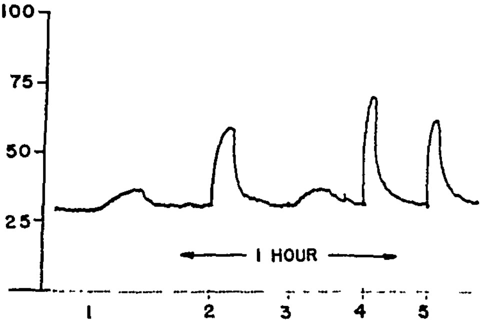

Fig. 3.—Contraction of aortic strip induced by urine from a patient with pheochromocytoma before and after removal of tumor. 1, Normal urine equivalent to 0.1 minute; 2, preoperative urine equivalent to 0.1 minute; 3, postoperative urine equivalent to 0.1 minute; 4, 0.3 μg norepinephrine; 5, 0.2 μg norepinephrine.

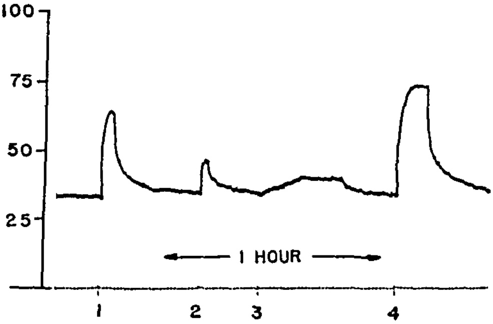

Fig. 4.—Ability of urine to enhance contraction produced by epinephrine. 1, 0.1 μg epinephrine; 2, 0.05 μg of epinephrine; 3, 0.1 ml. normal urine; 4, 0.1 ml. normal urine + 0.05 μg epinephrine.

There is a factor in urine which greatly enhances the contraction produced by epinephrine and norepinephrine (Fig. 4). This enhancement is similar to that produced by ethylenediaminetetraacetic acid (EDTA). Therefore, the catechol amine content cannot be assayed directly against these constrictor agents. In the quantitative assay of urines from patients with pheochromocytoma, norepinephrine with the addition of 0.5 mg. of EDTA to the bath has been used as a standard. It is also possible to substitute for the EDTA an amount of normal urine equivalent to that of the pheochromocytoma urine being assayed. The low catechol amine content of the normal urine would not interfere in assays of urines from patients with pheochromocytoma with high catechol amine values.

In Fig. 5 is shown an example of a quantitative assay of a urine from a pheochromocytoma patient. In this instance the urine being assayed was bracketed by known concentrations of norepinephrine; in other cases an exact match of the unknown and the standard was achieved.

In order to check the validity of the quantitative assay of urines which had a high catechol amine content, such urines were injected intravenously in a pithed cat to compare their pressor activity to that of norepinephrine.

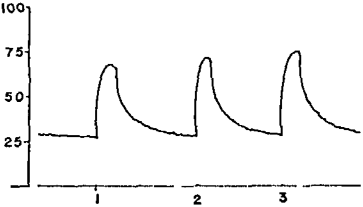

Fig. 5.—Quantitative assay of urine from patient with pheochromocytoma. 1, 0.5 mg. EDTA + 0.2 μg norepinephrine. 2, 0.04 ml. of pheochromocytoma urine. 3, 0.5 mg. EDTA + 0.25 μg of norepinephrine. Calculated value—5.6 μg per ml.

In Table I is shown a comparison of the aortic strip and pressor assay of 3 urines, 2 of high catechol amine content, and 1 which is definitely higher than normal but not as high as the proved cases of pheochromocytoma in our series. The assays of the more active urines were in good agreement by both methods. For the assay of these urines in a cat amounts of urine equivalent to only 0.17 to 0.27 minute were required to produce rises in arterial blood pressure of 38 to 24 mm. Hg.

TABLE I. COMPARISON OF THE AORTIC STRIP TECHNIQUE WITH DIRECT PRESSOR ASSAY IN CATS FOR THE DETERMINATION OF THE CATECHOL AMINE* CONTENT OF UNFRACTIONATED URINE

PATIENT	STRIP ASSAY (μG/ML.)	PRESSOR ASSAY (μG/ML.)
M. C.	5.7	5.0
M. S.	0.8	0.9
H. T.	1.0	0.27

*Expressed as norepinephrine.

With the urines of a lower catechol amine content, the pressor assay gave a much lower value than that obtained on the strip. An amount of urine equivalent to 1.85 minutes gave only a 14 mm. rise in blood pressure. With the larger amount of urine required, the lower value obtained in the pressor assay is probably due to the presence of kallikrein, an enzyme in urine which acts on the alpha-2 globulin fraction of plasma to form a depressor polypeptide, kallidin.[10]

Assays have been done on 560 urines from patients with elevated arterial blood pressure. Many of the urines were sent by mail from hospitals in other cities. In Table II are recorded the results of the quantitative assay of urines of 10 patients in whom the presence of pheochromocytoma has been proved by operation in 9 and by post mortem in 1. In 9 of these patients, the catechol amine content was more than 18 μg per hour. The highest value found was 396 μg per hour. One patient of the paroxysmal type, who had a positive histamine test, excreted only 8 μg per hour. The blood pressure of this patient at the time of urine collection was not recorded. After the removal of the tumor, only about 1 to 2 μg of catechol amines per hour were found in the urine.

TABLE II. URINARY EXCRETION OF CATECHOL AMINES, EXPRESSED AS NOREPINEPHRINE OF PATIENTS IN WHOM THE PRESENCE OR ABSENCE OF A PHEOCHROMOCYTOMA WAS VERIFIED BY OPERATION

PATIENT	μG/HR.	TUMOR + OR -	PHARMACOLOGICAL TESTS
M. C.	396	+	Regitine +
P. D. Y.	302	+	Not done
D. M.	144	+	Regitine +, Histamine -
M. S.	107	+	Regitine +
J. E.	98	+	Not done
F. G.	96	+	Regitine +
J. Sh.	37	+	Regitine +
M. B.	30	+	Not done
A. N.	18	+	Regitine +
P. B.	15-22	-	Regitine +
R. K.	15	-	Regitine +
A. F.	8	+	Histamine +

In addition to these patients 3 others were found who excreted 33 to 63 μg of catechol amines per hour. At this time these patients have not been subjected to an operation.

The average value of urinary catechol amines in 11 healthy laboratory workers was found to be 3.6 μg per hour with a range of 1.6 to 6.6 μg. Between these figures and the definitely elevated values found in the proved cases of pheochromocytoma during a phase of elevated pressure was a group of 6 patients with catechol amine concentrations in the urine which ranged from 12 to 22 μg per hour. Surgery was performed on 2 of these patients; in neither was an adrenal tumor found.

One of the patients (R. K.) had a blood pressure which varied between 110/70 and 180/120; she excreted 15 μg of catechol amines in 2 samples of urine submitted 2 months apart. When Regitine was given to her at a pressure of 180/120, there was a fall in blood pressure to 120/70. This patient weighed 235 pounds. Another patient (P. B.) with a fixed elevation of pressure, who had a positive Regitine test on two occasions, had urinary catechol amine values ranging from 15 to 22 μg in 3 urine samples. A tumor was not found in this patient on exploration of the abdominal cavity.

Five patients who did not have an elevated catechol amine content in their urine have been subjected to operation or have had a post mortem examination In none of these patients was a pheochromocytoma found

The value of adding Regitine to the bath to test whether the contraction produced by a urine being assayed is due to a catechol amine was demonstrated conclusively in a specimen obtained from a diabetic patient Two specimens of urine which were mailed to this laboratory caused a strong contraction of the strip The contraction was not abolished by Regitine* A kidney extract containing cathepsins destroyed the constrictor substance in this urine indicating the presence of a polypeptide, perhaps kallidin, which stimulates smooth muscle preparations[10]

The Effect of Therapeutic Agents on Assay—Chlorpromazine, hydralazine, and adrenolytic agents, when added to the aortic strip will prevent the constriction produced by epinephrine and norepinephrine Consequently, if patients are receiving these medications, there is a possibility that the excretion of these substances in the urine might interfere with the assay Two urines that were assayed abolished the contraction produced by norepinephrine One of the patients was receiving hydralazine Information was not received on the therapy of the other patient The urine collected 4½ hours after a Regitine test inhibited norepinephrine, that collected after 7½ hours had no such effect

Urine obtained from patients treated with reserpine, ganglionic blocking agents or barbiturates did not interfere with the assay

DISCUSSION

The estimation of catechol amines in urines by means of a spirally cut strip of rabbit aorta appears to be a useful adjunct in the diagnosis of pheochromocytoma It has these advantages Since such small amounts of unfractionated urine are required for the assay only a 1 to 2 hour urine sample need be collected If urines are adjusted to pH 3 0 or below, they can be stored in a refrigerator or in a frozen state until it is convenient to assay them The method is rapid With one strip urines of normal catechol amine content can be screened at the rate of 5 to 6 an hour The method has also proved useful as a confirmatory test in the diagnosis of pheochromocytoma

In most of the proved cases of pheochromocytoma reported in this paper, the catechol amine content of the urine was so high a quantitative assay was not necessary With values of 20 μg per hour or higher, false positives would be unlikely

On the other hand, it is quite possible that in patients of the paroxysmal type, an elevated content of catechol amines might not be found, particularly between attacks This possibility is emphasized by Litchfield and Peart on the basis of an interesting case they described recently[11] These authors concluded from the findings in this case that although the assay of catechol amines in amount of urine required, the lower value obtained in the pressor assay is probably due to the presence of kallikrein, an enzyme in urine which acts on the alpha-2 globulin fraction of plasma to form a depressor polypeptide, kallidin.[10]

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*The urine from this patient was also sent to Dr Marcel Goldenberg for a chemical assay for catechol amines This laboratory did not find an elevated content of epinephrine or norepinephrine

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A NEW TECHNIQUE FOR DIFFERENTIATION OF HEMOGLOBIN

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AND WOLF W ZUELZER, M D
DETROIT, MICH

THE detection of small amounts of a hemoglobin component in the presence of large amounts of another hemoglobin by means of paper electrophoresis is difficult This problem has been particularly evident with respect to the determination of fetal hemoglobin Chemically, the Singer 1 minute alkali resistance method[1] is unreliable at low levels, the more refined techniques are laborious and may give erroneous results in the presence of carbon monoxide hemoglobin[2] Also, the identification of alkali resistant hemoglobin by free electrophoresis is difficult and requires considerable care

The long known but little used agar gel electrophoresis medium seemed to us to offer some hope of separating the fetal component Gordon and associates[3] have demonstrated the separation of hemoglobin from ferritin, and Bussard and Perrin[4] have used agar as a supporting medium for serum protein separation The present method was first devised with the intent of providing an easy, clear cut method of separating fetal (alkali resistant) hemoglobin However, during the course of its development it proved to be valuable in detecting and quantitating both alkali resistant and other hemoglobins The method requires very little additional care than paper electrophoresis in operation and when used in conjunction with paper electrophoresis provides a means of distinguishing between hemoglobin D and hemoglobin S

The method has been applied to artificial and naturally occurring mixtures of hemoglobins A, F, S, D, and C The components A, F, S, and C gave characteristic separations (Fig 1), whereas D did not separate from A (Fig 5)

METHODS AND MATERIALS

Hemoglobin Solutions—Hemoglobin solutions were prepared by washing 3 times with isotonic saline, and lysing with distilled water and toluene The solutions were allowed to stand overnight in the refrigerator and then centrifuged at 3,000 r p m The clear hemolysate was then removed with a needle and syringe All samples were run as oxyhemoglobin The carboxyhemoglobin was first used but no difference in the pattern could be detected from that obtained with oxyhemoglobin samples

Paper Electrophoresis—The paper patterns were obtained using a veronal buffer, pH 8 6, ionic strength 0 05, in the apparatus described by Zuelzer and associates[5] Time of run was 16 hours at 7 Ma and 250 volts

Exchange Chromatography—The fetal hemoglobin preparation was carried out using IRC 50 resin and a citric acid citrate buffer as described in the method of Prins and Huisman[6]

From the Child Research Center of Michigan Detroit, Mich

This investigation was supported in part by a grant from the Rockefeller Foundation
Received for publication May 1, 1957

Solubility.—The solubility determinations were made using the method of Itano.[7]

Agar Electrophoresis.—The separation of hemoglobin using agar as a media was achieved as follows: a 1 per cent agar solution* was prepared using a citric acid-sodium citrate buffer, pH 6.5 and 0.05 M. (The buffer was prepared by dissolving 147 Gm. of sodium citrate [2 H_2O] in 600 ml. of H_2O, adjusting to pH 6.0 with concentrated citric acid solution, and diluting to a liter. This was the stock buffer solution. The buffer was prepared for use by diluting the stock solution, 1 part stock solution plus 9 parts water, and adjusting to pH 6.2 with citric acid solution.) The agar was heated until clear and then allowed to cool until just above the gelling point and then poured on a 4 by 10 inch thin glass plate which had filter paper wicks placed on each end and allowed to gel (approximately 25 ml. of solution is adequate). When the agar had cooled to room temperature samples of hemoglobin solution were introduced in the gel in the following manner: slits approximately ¾ inch long were made in the gel across the end of the plate with ¼ inch between the slits. A small strip of filter paper was then soaked with hemoglobin solution of approximately 10 Gm. per 100 ml. concentration and inserted in the slits. The entire plate was coated with a plastic by spraying with Krylon.† This is essential to prevent drying of agar during electrophoresis.

The plate was placed in a paper electrophoresis apparatus previously described[5] which contained the same buffer as used to make up the agar solution. A current of 20 Ma. and 350 volts (supplied by a Heath Kit) produced good separation in 16 hours.

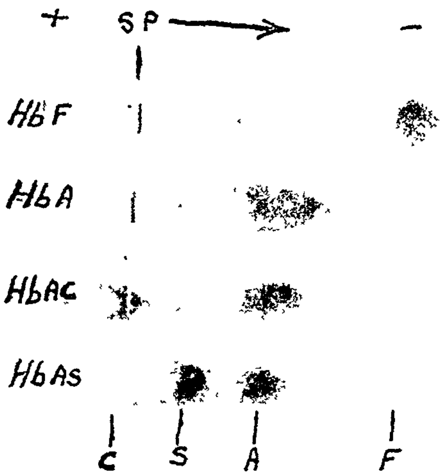

Fig. 1.—Typical agar plate pattern, with samples of hemoglobins F, A, AC, and AS.

The hemoglobins under these conditions migrate toward the cathode as would be expected at this pH. However, as can be seen in Fig. 1, the order of separation is not the same as that found in free electrophoresis at pH 6.2, since alkali resistant hemoglobin migrates fastest with hemoglobins A, S, and C following in that sequence. This is the reverse of the order found in free electrophoresis but very similar to the manner in which hemoglobins separate on the IRC 50 resin column of Prins and Huisman.[6] This order would indicate that the separations are due to a combination of adsorption and electrophoretic effects.

The agar plates may be stained by stripping off the plastic coating and staining with Amino-Schwartz 10B in a methyl alcohol, water, and acetic acid solution (50:50:5) for 20 minutes followed by careful washing with 2 per cent acetic acid solution. After staining and washing the plates are dried at room temperature The agar adheres to the plate like photographic emulsion and gives a low background when read in a densitometer Quantitation by this means yields results comparable to that obtained on paper

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*Bacti-Agar, Difco Laboratories, Detroit, Mich.

†Manufactured by Krylon, Inc., Norristown, Pa.

RESULTS

Fig 1 illustrates the typical separations obtained using hemolysates from known samples of F, A, AC, and AS hemoglobins The F sample (cord blood)

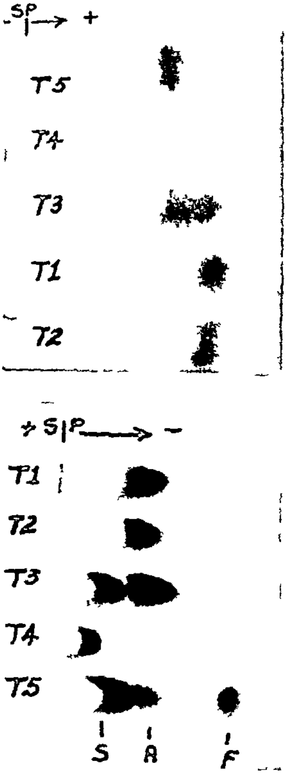

Fig 2.—Comparison of (A) paper electrophoresis and (B) agar electrophoresis in a family study of sickle cell thalassemia

had been found to have 87 per cent alkali resistant hemoglobin As can be clearly seen the pattern of the F sample demonstrates the presence of a small amount of A which paper electrophoresis had failed to show The A, AC, and

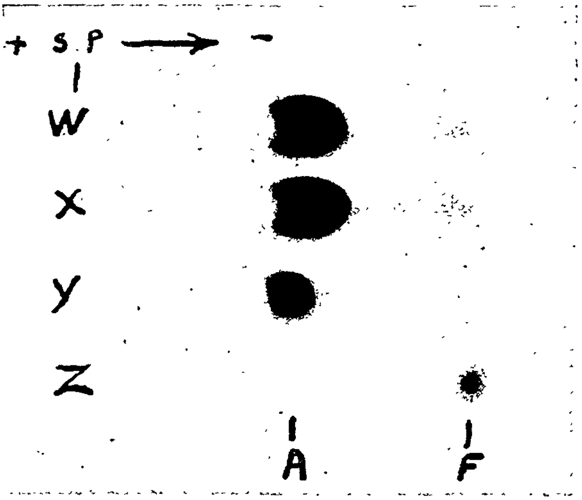

Fig. 3.—Illustration of effect of decreasing amount of hemoglobin introduced on agar plate.

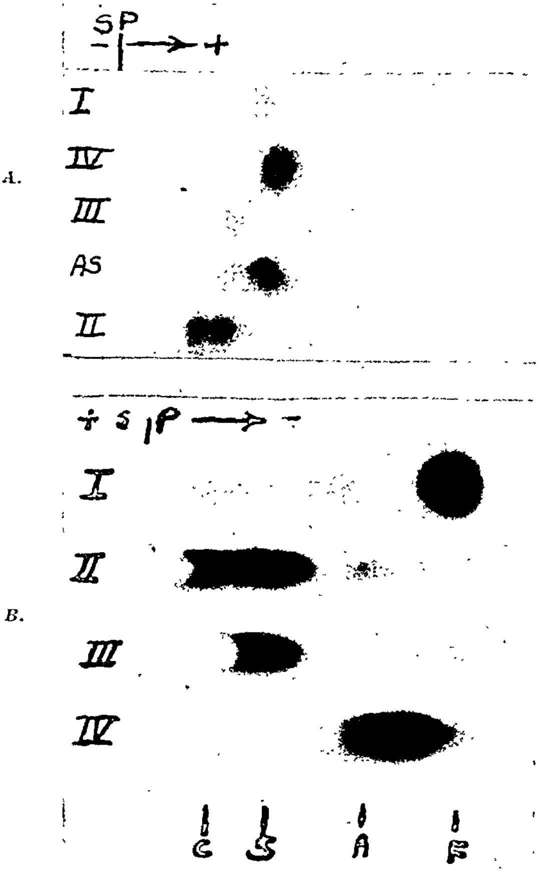

Fig. 4.—Comparison of small components using (*A*) paper electrophoresis, and (*B*) agar plate method.

AS samples contained between 1 and 2 per cent alkali resistant hemoglobin As can be seen in the illustration, A S, and C are clearly separated and a spot appears in the F position with an intensity comparable to the percentage of alkali resistance obtained by the chemical method

A second faint spot, visible on agar in the A, AC, and AS samples just ahead of the A position has not been identified as yet but was present in many samples examined In the A and AC patterns there was also an increased density in the S position This was reproducible and occurred in only a few specimens The authors have as yet been unable to determine whether this represents sickle hemoglobin another hemoglobin with a mobility similar to sickle, or an artifact However with the increasing reports in the literature[8, 9] of minor components in normal hemoglobins these small amounts of hemoglobin or hemoglobin like substances merit attention

Fig 2 demonstrates the use of the agar method in a family with sickle thalassemia disease in two members

Case Report—T4 was a 12 year old boy whose blood sickled on smear and who had a large number of target cells His hemoglobin was 2 Gm per 100 ml and his R B C was 800,000

T5 was a 16 year old girl whose blood sickled on smear and had a large number of target cells Her hemoglobin was 9 Gm per 100 ml and her R B C was 4 100,000, hematocrit 31 per cent, and reticulocytes 2 2 per cent

The blood of the father (T3) was that of a typical sickle trait, the blood of the mother (T2) manifested thalassemia minor

Comparing agar patterns with paper patterns it was seen that T4 and T5 on paper showed predominantly S with only an indication of F, while on agar T5 clearly showed A and F, and T4 showed a faint indication of A with a distinct spot for F The hemoglobin F is very clearly separated Chemically T5 had 10 per cent, and T4 15 per cent alkali resistant hemoglobin This may not appear so from the illustration, but the T4 hemolysate was very weak (3 Gm per 100 ml) as compared to T5 whose hemolysate was 10 Gm per 100 ml This was unavoidable as T4 was severely anemic T3, the father, clearly demonstrates the presence of A and S with a small amount of F (1 per cent alkali resistant) The mother (T2) and sibling (T1) possessed only A with less than 1 per cent alkali resistant Again good agreement existed between the intensity of the F spot and alkali resistance determined chemically

The concentration of hemoglobin in the hemolysate was found to be significant Fig 3 illustrates the effect of lowering the hemolysate concentration Sample W was A with 2 per cent alkali resistant hemoglobin and an hemolysate of 11 Gm per 100 ml , sample X was A with 1 8 per cent alkali resistant hemoglobin with an hemolysate of 6 Gm per 100 ml , sample Y was A with 2 9 per cent fetal, hemolysate 3 Gm per 100 ml , sample Z was A with 56 per cent alkali resistant hemoglobin and an hemolysate of 2 Gm per 100 ml

It can be seen from this illustration that variation of the hemolysate concentration from 6 to 10 Gm per 100 ml causes little difficulty in the recognition of minor components However when the concentration was dropped to

3 Gm. per 100 ml., the F component was not discernible. The necessity of maintaining the hemoglobin concentration at the proper level is more clearly demonstrated in sample Z where the A component is barely discernible. It should also be noted that with increasing amounts of hemoglobin, the spot increases in the direction of movement. Note the difference between the A component in samples Y and X. If the concentration is increased too much or if too large an amount of hemolysate is introduced onto the plate, an "overload" pattern develops in which a single spot migrates from the A to the F position and all intermediate details are obliterated (see Fig. 4, sample IV).

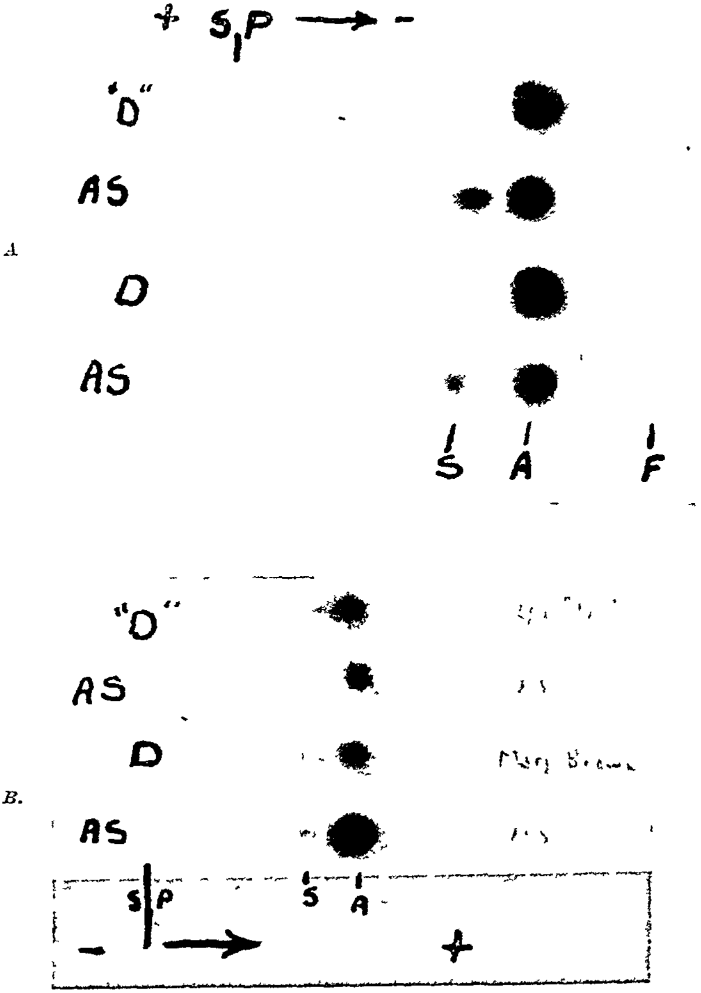

Fig. 5.—Illustration of differences in behavior of hemoglobin D and hemoglobin S on (*A*) agar, and on (*B*) paper electrophoresis

Fig. 4, sample I, obtained from a newborn infant, showed the presence of A- and C-like components, demonstrated on agar when not detectable on paper. This sample contained 94 per cent alkali resistant hemoglobin when tested by chemical methods and appeared as a single spot on paper The confirmation of the actual presence of A and C must await the disappearance of the fetal hemoglobin and the appearance of the adult hemoglobin in this infant

Sample II was shown on paper to contain S and C, the alkali resistance tested chemically was 1 6 per cent and the sickle preparation was positive When tested on agar the presence of distinct spots in the A and F positions was noted

Sample III appeared as a single component, S, on paper The patient's erythrocytes sickled on smear The hemoglobin showed 1 per cent alkali resistance No signs of thalassemia were manifested On chemical and genetic grounds this individual was thought to have sickle cell anemia When sample III was analyzed on agar a faint spot was visible in the A position in addition to a spot in the F position Thus, it would appear from samples II and III that an A like component does occur in the SC combination and that apparently pure S may also be accompanied by an A like component (as in sample III)

Sample IV was *hemoglobin A and illustrated what happened when the* agar is overloaded with hemoglobin

Fig 5 demonstrates the behavior of D As was clearly shown by the paper *patterns,* samples D and "D" separated into two spots in the A and S positions Both samples were likewise shown to have peaks in the A and S positions in free electrophoresis using pH 6 5, 0 1 ionic strength, cacodylate buffer The samples were tested for solubility by the Itano method and found to have solubilities identical with that of an AD mixture As can be seen in Fig 5, on agar a single spot in the A position was found D, thus, did not separate from A However, S, in an AS combination, did clearly separate

This method in conjunction with paper electrophoresis provides a means of identifying D in the presence of S without testing for solubility

The method has also been employed on samples of hemoglobin AG It was found that G separated in a position just slightly ahead of S Unfortunately illustrations of this effect are not available It is hoped that more samples of the now known types of hemoglobin will become available to us for testing by this technique

DISCUSSION

The agar technique has been employed for the detection of both small and large amounts of alkali resistant hemoglobin The concentration detected varied from less than 1 per cent to 94 per cent The ability of the method to detect very low concentrations of alkali resistant hemoglobin and yet maintain good agreement with chemical determinations make the method a useful tool In the development of the method however, its ability to separate major components, and, on the other hand, to show the presence of small components of other hemoglobin like substances has further increased its value Indeed in the light of the possible genetic implications, these findings of an A like substance in the SC combination and in the S homozygote, may be the method's most valuable aspect

The additional demonstration of the failure of the method to separate D from A provides, when taken in conjunction with paper electrophoresis, a means other than the solubility for demonstrating the presence of this hemoglobin.

SUMMARY

The technique as described here has provided a means of clearly demonstrating the presence of alkali resistant hemoglobin electrophoretically. We are also able to separate hemoglobins A, S, and C in a manner comparable to that obtained by paper electrophoresis. The method is unable to separate D from A but when used in conjunction with paper electrophoresis and the sickling technique thus provides another characterization of D.

At least one other component is demonstrated in normal blood which as yet has not been identified. The appearance of minor components in normal and abnormal blood in the position of known hemoglobins and in intermediate positions awaits further investigation and application of this method.

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THE LABORATORY DETERMINATION OF PEPSIN IN GASTRIC JUICE WITH RADIOACTIVE IODINATED ALBUMIN

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THE determination of pepsin in gastric juice is not a routine laboratory procedure. Occasionally pepsin determinations have been performed to estimate expected progress in the healing of known peptic ulcer.[1] Little clinical attention, however, is given to gastric pepsin currently since the main attack of ulcer therapy is in neutralization of the free acid in the stomach.

When pepsin determinations are performed in the laboratory today, the hemoglobin method is the procedure most frequently used. This method usually involves diluting the gastric juice sample in order to decrease the pepsin concentration to the sensitivity range of the test. This dilution factor introduces a variable in peptic activity that must be taken into account in estimating the concentration of the enzyme.[2, 3]

A simple, direct method of measurement of peptic activity on routine gastric analysis samples can be performed with radioactive I^{131}-labeled albumin. This procedure is described below. It compares favorably with the established hemoglobin method of pepsin assay and it provides a clinical estimate of pepsin concentration in the gastric juice of the patient being tested.

MATERIALS AND METHODS

Gastric juice is obtained in the usual manner of gastric analysis in the fasting patient. A Levin tube is passed and the fasting residual is removed and saved. Thereafter, four 15 minute fractions of gastric juice samples are collected by constant hand suction. This series of 5 specimens constitutes the basal gastric analysis. The juice is centrifuged to spin out solid particles and mucus, and the volumes are measured

One milliliter of radioactive iodinated albumin† of known specific activity of approximately 0.03 μc/ml. and 1 ml. of 6 per cent bovine albumin as a carrier are added to an 8 ml. test tube. The pH is adjusted to 2 by the addition of 3 ml. of Sørensen's buffer. (Sørensen's buffer: 7.51 Gm. of glycine and 5.85 Gm. of NaCl in one liter of water added to one liter of 0.1 N HCl.) One milliliter of the clear centrifuged gastric juice is then added and the mixture is incubated at approximately 37° C. for 15 minutes.

Following incubation, 1 ml. of 50 per cent trichloroacetic acid (TCA) is added The precipitate is centrifuged and the clear supernatant is decanted into tube 1. The remaining precipitate is washed once in 5 ml. of 7 per cent TCA, spun down, and the solution decanted into tube 2. (Seven per cent TCA is used in this step since it compares with the concentration in the original sample when 50 per cent TCA has been added to it.) The fixed precipitate is dissolved in the third tube with 0.2 N NaOH and all 3 tubes brought up to the same level and counted for one minute in a Nancy Wood scintillation counter. The second tube containing the wash solution is counted 3 minutes because of its low activity. The sum of the activity of the supernatant fluids is a measure of enzymatic action on the radioactive iodinated albumin substrate. The per cent of activity is calculated by dividing the total count, including the count of the precipitate, into the sum of the counts of the supernatant fluids and multiplying by 100. A standard curve of reference is made by the use of 2 X crystallized pepsin* with concentrations extending over a range of 0.1 mg. through 5.0 mg. The activity of the unknown can be read directly from this (Fig. 1). The per cent of recovery is synonomous with the per cent of activity.

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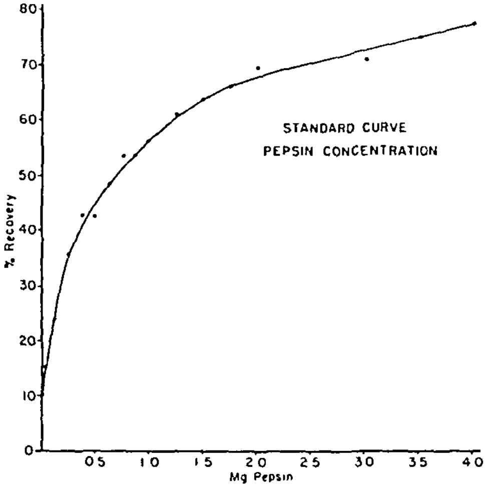

Fig. 1.—Standard curve with use of 2 X crystallized pepsin.

Since pepsin concentrations in gastric juice are usually high, the procedure can be considerably shortened without interfering with the validity of the test by performing only one step after incubation. In this short method, trichloroacetic acid is added after incubation and the supernatant fluid from centrifugation is counted. A blank of 1 ml. of iodinated albumin brought to an equal volume serves as the control and the per cent activity is calculated from this. (Table I.)

Table I. Comparison of Results of Detailed Method With Abbreviated Method

DETAILED METHOD OF PEPSIN ASSAY WITH RADIOACTIVE IODINATED ALBUMIN				ALTERNATIVE METHOD (MG. PEPSIN/ML. GASTRIC JUICE)
SAMPLE	PER CENT ACTIVITY	MG. PEPSIN/ML. GASTRIC JUICE	PER CENT ACTIVITY	
1	53.0	0.85	52.8	0.84
2	42.4	0.43	43.5	0.44
3	41.7	0.43	41.7	0.43
4	23.5	0.13	23.5	0.13
5	39.8	0.38	40.8	0.39
6	15.3	0.05	15.3	0.05
7	30.0	0.19	30.0	0.19
8	48.9	0.67	50.2	0.71
9	30.3	0.19	30.7	0.20
10	39.6	0.36	39.3	0.35
11	53.8	0.88	52.8	0.84
12	41.5	0.43	43.2	0.44
13	36.2	0.27	34.9	0.25
14	60.3	1.24	60.3	1.24
15	28.8	0.18	27.5	0.17

RESULTS

This procedure as described using 1 ml. of gastric juice has been used in hundreds of determinations of pepsin in gastric juice samples obtained by routine

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*Nutritional Biochemical Corporation, Cleveland, Ohio.

Table II Basal Gastric Analysis Study Using One c.c. of Gastric Juice From Each 15 Minute Specimen

TUBE NO	SPECIMEN	VOLUME (ML.)	FREE HCL (CLINICAL UNITS)	TOTAL ACID	MEQ PER VOLUME	TOTAL COUNTS	C.P.M. OF SUPERNATE AND WASH	PER CENT ACTIVITY	PEPSIN (MG./ML.)
1	Fasting	13	18.0	30.0	0.075	6,774	2,031	30.4	0.19
2	Basal—15 min	8	64.0	72.0	0.186	7,270	3,480	47.9	0.61
3	30 min	9	60.0	68.0	0.197	6,678	3,449	51.0	0.75
4	45 min	24	32.0	42.0	0.280	6,653	3,710	55.8	0.97
5	60 min	25	46.0	54.0	0.418	6,671	3,688	55.4	0.96

basal gastric analysis. A typical example of one such patient study is shown in Table II. Some comparative values with the hemoglobin method on "unknowns" of gastric juice are shown in Table III.

TABLE III. COMPARISON OF PEPSIN "UNKNOWNS" BY HEMOGLOBIN AND ISOTOPE METHODS

NUMBER OF "UNKNOWN"	HEMOGLOBIN METHOD	I^{131} METHOD
1	0.05 mg. pepsin	0.04 mg. pepsin
2	0.08 mg. pepsin	0.06 mg. pepsin
3	0.10 mg. pepsin	0.07 mg. pepsin
4	0.75 mg. pepsin	0.79 mg. pepsin
5	0.12 mg. pepsin	0.08 mg. pepsin

TABLE IV. INCREASING SENSITIVITY WITH DECREASE IN CONCENTRATION OF 6 PER CENT ALBUMIN CARRIER

2.5 MG. CRUDE PEPSIN WITH VARYING AMOUNTS OF 6 PER CENT ALBUMIN CARRIER	PER CENT ACTIVITY
1.0 ml.	33.7
0.9 ml.	34.4
0.8 ml.	39.8
0.6 ml.	45.9
0.5 ml.	46.9
0.3 ml.	55.2
0.1 ml.	60.2

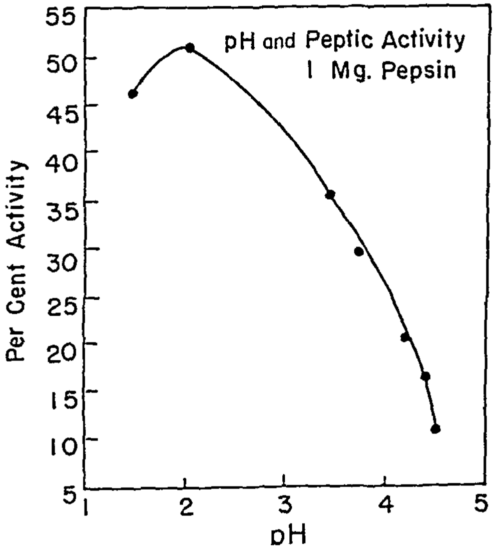

Fig. 2.—Influence of pH on peptic activity as determined by the isotope method.

DISCUSSION

Gastric juice usually has a high pepsin concentration which may not be appreciated clinically. The measured enzyme activity in milligrams of pepsin of one cubic centimeter of gastric juice determined by the method described and multiplied by volume provides an estimate of the total enzyme output in the 15-minute sample being tested. Standardization of the pepsin curve used in this method is simple and values are read directly from percentage of activity. The procedure can be applied to any routine clinical gastric analysis sample.

Since enzyme activity in gastric juice is in a concentration that usually requires dilution of the juice for the hemoglobin method, it is advantageous to perform determinations on an undiluted sample If increased sensitivity of the test for smaller amounts of pepsin comparable to the hemoglobin method is desired, this can be achieved by decreasing the concentration of the bovine albumin carrier as shown in Table IV

The pH of gastric juice in vivo is found to vary from time to time throughout the day in fractional samples and enzyme activity in such instances varies with this change in pH Adjusting to a constant pH, however, reveals the potential peptic activity of the juice being sampled Reference to peptic activity at increasing pH as shown in Fig 2 reveals peptic activity at pH values higher than 2 and as high as 3 5 to 4 A pH range this high necessarily means the absence of free acid in the stomach, but this does not mean the complete cessation of peptic activity in the stomach This may have some influence on the healing of peptic ulcer It has been reported that pepsin is formed from a solution of crystalline pepsinogen after incubation at a pH as high as 4 65,[4] and according to Somers,[5] it forms from pepsinogen at a pH of 5 1 An in vivo pH of 3 or higher, therefore, does not totally eliminate peptic activity

There seems to be no clinically effective pepsin inhibitor used in peptic ulcer therapy today Reported suppression of activity is most probably due to an increase in pH with some concomitant decrease in peptic activity[4] For complete inhibition of peptic activity by antacid management a constant gastric pH above 4 65 would seem to be necessary Achievement of this high a gastric pH is not the case in the usual ulcer management employed today

SUMMARY

A simple method for direct measurement of gastric juice peptic activity is described

The procedure can be used in all routine gastric analysis samples and may be of clinical interest in providing an estimate of total gastric pepsin output

Clinically significant peptic activity may be present in gastric juice in the absence of free acid

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THE USE OF CHICKEN ANTISERUM FOR THE RAPID DETERMINATION OF PLASMA PROTEIN COMPONENTS

II. THE ASSAY OF HUMAN SERUM OROSOMUCOID

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INTRODUCTION

THE rapid turbidimetric procedures developed for the measurement of human serum albumin and gamma globulin by the use of chicken antisera[1] have now been extended to the assay of a number of other human proteins. These include fibrinogen, transferrin, a beta lipoprotein, a heat labile alpha-2 glycoprotein of large molecular weight,[2] two small molecular weight alpha-2 glycoproteins,[3] and orosomucoid.[4] A major effort has been devoted to the determination of orosomucoid whose serum levels are being followed serially in a large number of cancer patients. The orosomucoid-chicken anti-orosomucoid system was found to be a somewhat different immunochemical system than those previously studied.[1] The present paper describes the optimal conditions for carrying out the orosomucoid assay and compares the serum levels of orosomucoid in normal and cancer subjects.

Orosomucoid* is a low molecular weight protein of acid isoelectric point[5-8] which resists coagulation by heat and precipitation by acids such as sulfosalicyclic or perchloric but which does precipitate in phosphotungstic acid. It is a major constituent of the "seromucoid" or serum "mucoprotein"[9] fraction and exhibits a polarographic wave. Serum mucoprotein concentrations, as estimated chemically, increase in a number of pathologic states and have been extensively investigated in cancer.[5, 10-14] Orosomucoid, in contrast to the seromucoid fraction, is homogeneous both chemically[6, 7] and immunochemically[15] and probably represents a single biologic entity. Consequently, it was felt that the specific measurement of orosomucoid might provide a more meaningful index for the evaluation of clinical state than the chemical estimation of a heterogeneous fraction.[16]

MATERIAL AND METHODS

Orosomucoid was prepared from fraction (Fr) VI† of pooled human plasma by a modification of the method of Weimer and associates.[6] In a typical preparative run 10 Gm. of

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*Synonyms for this protein are M-1 protein, MP-1 protein,[6] and acid glycoprotein.[7]

†This fraction was generously provided to us by the Blood Products Section of the Michigan Public Health Service, Lansing, Mich

lyophilized Fr VI were dissolved in 100 ml of distilled water Saturated $(NH_4)\,SO_4$ (4 1 M) was added to a concentration of 2 57 M The precipitate was removed by centrifugation and the pH lowered to 4 9 After overnight standing at 4° C the next precipitate was centrifuged off The pH was lowered to 3 7 and the $(NH_4)\,SO_4$ concentration to 2 05 M The new precipitate that formed after overnight standing at 4° C was removed Solid $(NH_4)\,SO_4$ was then added to the solution until full saturation was reached The pH was maintained at 3 7 After several days standing at 4° C the crude orosomucoid precipitate was recovered by centrifugation The packed precipitate was dissolved by the addition of 27 ml of distilled water To the 45 ml of solution that resulted 78 ml of sat $(NH_4)\,SO_4$ was added The pH was adjusted to 4 9, and the precipitate that formed after overnight standing at 4° C was centrifuged off The pH of the supernatant was lowered to 3 7 This time the $(NH_4)\,SO_4$ concentration was not reduced Processing was then carried through as before The final orosomucoid precipitate after recovery by centrifugation was dissolved, exhaustively dialyzed against distilled water and lyophilized to yield 2 Gm of material The centrifugation steps were all carried out in the cold at 8 000 to 12,000 r p m * The preparation had less than 1 per cent contaminants as determined by antisera to albumin and to the alpha 2 glycoproteins of Fr VI It was also homogeneous by electrophoresis and ultracentrifugation and possessed the expected physicochemical constants † Orosomucoid preparations of less purity could be detected during the recycling by the greenish color of the pH 3 7 supernatants Cautious fractional addition of sat $(NH_4)\,SO_4$ then sufficed to salt out the contaminants

During the current studies, 31 healthy hens, 6 to 12 months of age and of various breeds, underwent immunization Antibody responses were quite variable by each of the injection schedules followed The highest percentage of usable sera were produced by a schedule in which each bird received intramuscularly into 4 spots an injection of 200 mg of orosomucoid well mixed with aluminum hydroxide gel Ten days later the blood was collected and processed according to the procedure of Silberberg and associates[1] About 30 per cent of the birds yielded negative serum Efforts are continuing to find more effective injection procedures ‡

The specificity of each antiserum was evaluated by carrying out flocculation reactions and Oudin tests[3] with Fr VI and its various components orosomucoid, albumin, and two purified alpha 2 glycoprotein preparations § A number of the Oudin tests were carried out in 13 per cent NaCl instead of 8 per cent Antisera reacted at 13 per cent NaCl were first incubated for 2 hours at 38° C after addition of the proper amount of NaCl The residual fibrinogen, having flocculated, was then removed by centrifugation Antisera that were completely specific for orosomucoid yielded only one band in the Oudin tests and only precipitated with Fr VI and orosomucoid Potency was estimated in a qualitative manner by noting the degree of flocculation when 1 10 and 100 mcg of orosomucoid in 0 1 ml aliquots were mixed, respectively, with 0 2 ml portions of the antiserum dilution (1 to 2 after processing) Antisera showing comparable potencies and specificities were pooled together

Biuret determinations, employing the reagent of Cornall and associates,[18] were carried out as previously described[1] Hexose and seromucoid determinations were performed according to methods outlined by Winzler[4]

RESULTS

Effect of NaCl Concentration —The effects of NaCl concentration on avian precipitin systems have been intensively investigated[19 21] Specific precipitation was found to increase on raising the NaCl concentration from 1 to 8 per cent. With antialbumin and antigamma globulin systems further elevations above 8 per cent caused a broadening of the zone of maximal precipitation into the far antigen excess zone but did not markedly increase the precipitation at equivalence. The difference in precipitation on varying salt concentration was dependent on the concentration of reactants—the higher the salinity the smaller the inhibition of precipitation on lowering the concentration of reactants.[19, 20]

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* A Serval or high speed head No 855 of the International proved convenient for this purpose

†We are indebted to Dr Karl Schmid for performing the electrophoretic and ultracentrifuge analyzes of this preparation

‡Procedures employing combined intermuscular (with adjuvant) and intravascular injections appear to hold promise

§These were supplied to us by Dr Karl Schmid

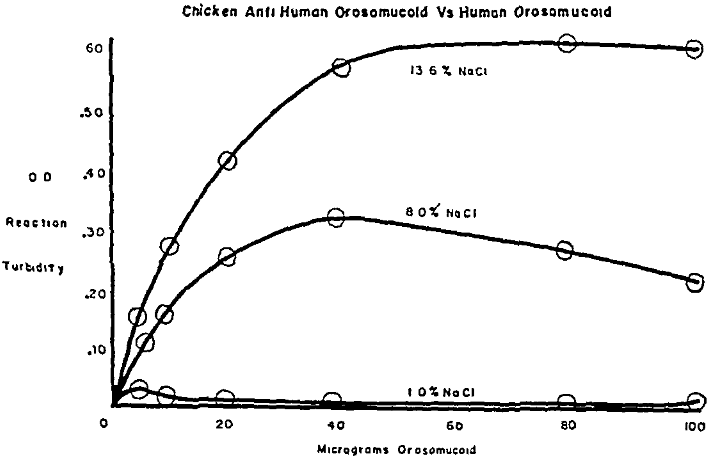

Fig 1 —Effect of NaCl concentration on precipitate formation-chicken antiorosomucoid serum (C-17) plus orosomucoid, 0 1 ml. antigen + 0 4 ml. ¼ C-17. One hour incubation at 38° C Reaction turbidities recorded in Beckman spectrophotometer set at 450 mμ.

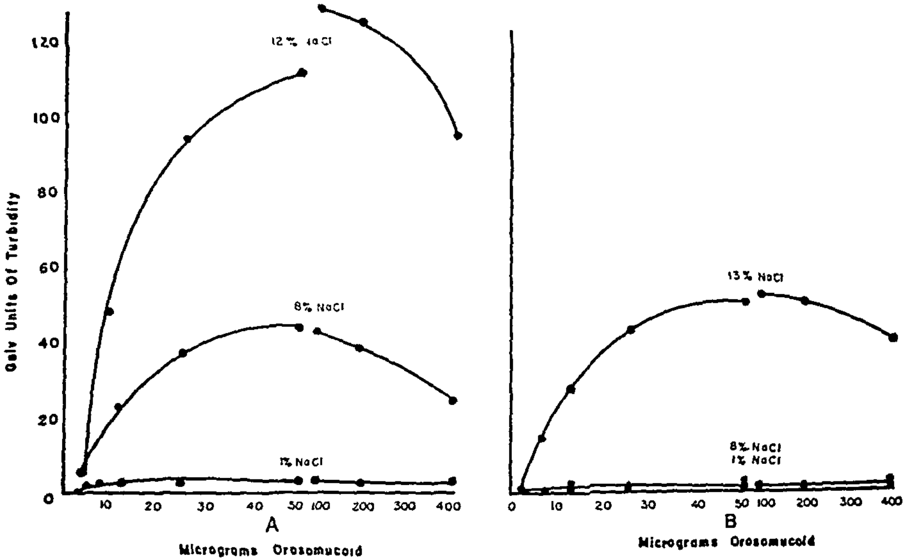

Fig 2.—Effects of NaCl concentration and volume on precipitate formation-chicken antiorosomucoid serum (P-17) plus orosomucoid *A*, 5.5 ml. reaction volume containing 0 5 ml P-17; 20-minute incubation at 38° C. *B*, 5 5 ml reaction volume containing 0 25 ml. P-17; 45-minute incubation at 38° C. Nephelometric determination of turbidity

It occurred to us that the chicken antiorosomucoid-orosomucoid system might show these salt effects to a more pronounced degree and that it might be advantageous to react this system at 12 to 15 per cent NaCl instead of 8 per cent Figs 1 and 2 demonstrate this to be the case Precipitation failed to develop in 1 per cent NaCl and was much less at 8 per cent than at 12 or 13 6 per cent NaCl This was true in the antibody excess zone as well as the equivalence and antigen excess zones

At the high dilution of reactants employed in the nephelometric method the salt effects were accentuated After the initial incubation at 38° C the precipitin reactions in 12 to 13 per cent NaCl were essentially complete except in the far antibody excess zone There increased turbidity developed after overnight standing The slower rate of precipitation at small concentrations of orosomucoid was more marked in 8 per cent NaCl than in 12 to 13 per cent and extended to larger concentrations of the antigen Indeed, with half the quantity of antiserum (see Fig 2, *B*) detectable precipitation in 8 per cent NaCl only developed after overnight standing In 1 per cent NaCl precipitation was insignificant even after 5 days incubation at 5° C

Procedures for the Assay of Orosomucoid—Based on the above results the following orosomucoid assay method was set up with an antiserum (P 17)* exhibiting high potency and specificity A 0 5 ml of the antigen solution at 13 per cent NaCl was pipetted into a test tube Then 0 5 ml of a 3 5 dilution of P 17 adjusted to 13 per cent NaCl, was introduced After 15 minutes incubation at 38° C, 4 7 ml of 14 per cent NaCl was added These two additions were effected in each case with an appropriate automatic Cornwall syringe to permit rapid mixing of the reagents The contents of the tube were transferred to a calibrated cuvette, and turbidity was read in the Coleman model 7 photonephelometer The standards varied from 0 mcg orosomucoid to 25 mcg, the 0 tube providing the reagent blank upon which the instrument was set at zero galvanometer units A 0 5 ml of 1 100 dilution of human serum in 13 per cent NaCl was chosen for sample assay This amount permitted ready quantitation of the elevated orosomucoid values occurring in certain pathologic states, normal values falling on the lower quarter of the calibration curve The galvanometer units of turbidity of typical standard tube reactions are presented in column B of Table I

An orosomucoid assay which required only one half to one third the amount of antiserum used in the nephelometric procedure was also worked out with the Beckman D U spectrophotometer† The reactions were developed in 0 5 ml volume at 13 per cent NaCl in 10 by 75 mm test tubes After incubation at 38° C for 1 hour, by which time flocculation was complete, each reaction tube was shaken and its contents transferred to a microcell requiring a minimum volume of 0 35 ml The turbidity as optical density units (O D) was read against a reagent blank consisting of antiserum plus 13 per cent NaCl placed in a companion microcell The readings were taken at 340 mμ with the ultraviolet source of light Under these spectrometric conditions the largest turbidity readings could be obtained with the least interference from the protein and hemoglobin contents of the reaction mixtures Standards were varied from 0 to 12.5 mcg. orosomucoid, and the readings at the top portion of the calibration curve were 0.400 to 0.500 O.D. units. The routine quantity of human serum chosen for assay was 0.002 ml. (either 0.1 ml. of a 1:50 dilution or 0.2 ml. of a 1:100 dilution in 13 per cent NaCl).

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*A pool of 4 antisera of similar characteristics each at the routine 1 2 dilution in 8 per cent NaCl

†With other sensitive laboratory spectrophotometers such as the Bausch and Lomb Spectronic 20 and the Universal Coleman comparable assay procedures can be worked out with suitable microcells

TABLE I. EFFECT OF VOLUME AND TIME ON THE STANDARD TUBE REACTIONS EMPLOYED IN THE NEPHELOMETRIC METHOD

OROSOMUCOID (MCG.)	1 ML. INITIAL REACTION VOLUME						5.7 ML. INITIAL REACTION VOLUME
	A		B		C		D
	7.5" 38° C. + 45" 25° C.		15" 38° C. + 45" 25° C.		30" 38° C. + 45" 25° C.		60" 38° C.
0	0	0	0	0	0	0	0
3.13	4.5	9	13	13	14	13	3
6.25			21	21.5			17
12.5			40	39			36
25.0	61.5	62	63	63	63.5	62.5	54

A = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl); incubate 7.5 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

B = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl); incubate 15 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

C = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 0.5ml. 3:5 P-17 (at 13 per cent NaCl); incubate 30 min. 38° C., then add 4.7 ml. 14 per cent NaCl.

D = 0.5 ml. orosomucoid (at 13 per cent NaCl) + 4.7 ml. 14 per cent NaCl + 0.5 ml. 3:5 P-17 (at 13 per cent NaCl).

During the past two years over 1,500 human sera have been assayed for orosomucoid. The above turbidimetric procedures have been employed in the assay of the last 800 samples. To help insure uniformity on going from one antiserum to the next, the orosomucoid value of a large pool of human serum from patients with elevated values was calculated by repeat analyzes with our best reagents, a highly potent and specific antiorosomucoid serum (P-17) and a highly pure orosomucoid preparation (oroso 8). The calibrated serum pool (PCS) was divided into small vials and stored in a deep-freeze. As needed, portions have been thawed to provide standards for the orosomucoid assay.

The calibration curves obtained by reacting P-17 with PCS and oroso 8 were very similar, but not identical. As constructed from the orosomucoid value (250 mg. per cent) assigned to PCS, the curves intersected each other at their mid-portions. The divergence of the 2 curves was found in some cases to be more pronounced with other orosomucoid preparations and on reacting different antisera. These data are illustrated in part by Fig. 3. It can be seen that oroso 16 afforded less precipitation on a comparable weight basis than oroso 8. Oroso 16 was prepared by extensive refractionation of a heterogeneous preparation showing a lower salting out range in $(NH_4)_2SO_4$ solutions than oroso 8. The only contaminant detected immunochemically in oroso 16 was a trace of alpha-2 glycoprotein.

Table II demonstrates that with PCS as the standard the same sample assay values within experimental error were obtained by the various antiorosomucoid sera employed. Some of these sera (e.g., P-30) lacked complete specificity and others (e.g., C-124) exhibited inhibition of precipitation in the far antibody excess zone. The human serum samples could be stored for as long as

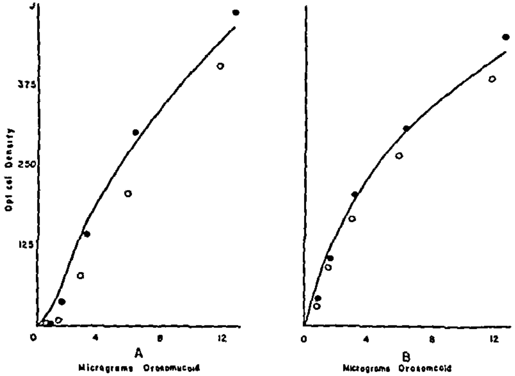

Fig 3 —Compari sera with anti
body excess zone rea(PCS calibration
curve Solid circle = *A*, 0 2 ml Ag +
0 3 ml Ab (1 2 52 C- *B*, 0 2 ml Ag +
0 3 ml. Ab (1 2 P-17) action turbidities
recorded in Beckman mμ

Table II. Identity of Sample Assay Values by Different Antiorosomucoid Sera

SAMPLE NO.	C 17* (MG. %)	P 17 (MG. %)	P 18 (MG. %)	P 30† (MG. %)	C 124‡ (MG %)
532	120				115
621	202				203
644	140				139
682	235				227
790		70			80
853		263	243		
856		193	181		
858 (N)§		57	50		
921		125			125
935		72			101
1,074 (N)		66		70	
1,076		235	250		227
1,079		238	225		255
1,082 (N)		78		80	
1,106		*83*			*80*
1,200 (N)		96	88		93
1,202 (N)		67		70	68
1,203 (N)		75		60	
1,212 (N)	–		70		56
1,239			210		200
1,249			325		350
1,252			80		85
1,301				145	151
1,313				105	105
1,334				176	160
1,383				345	353
1,423				200	235
1,441				215	212
1,480				220	195

*The current turbidimetric method was being evolved at the time that C-17 was employed The standard, then, was made up from an individual calibrated cancer serum of high orosomucoid content. It served as the forerunner for PCS

†P-30 yielded a weak reaction with an alpha-2 glycoprotein in addition to its strong reaction for orosomucoid

‡The determinations with C-124, our most recent antiserum, were carried out almost a year *after C-17 had been consumed in testing*

§(N) designates a sample from a normal donor.

10 months in the liquid state at -2° C. without apparent effect on the orosomucoid assay. The duplication error of the method was ± 9 per cent in the hands of laboratory technicians.

The reliability of the orosomucoid assay was further investigated by adding known quantities of purified orosomucoid to solutions of other serum proteins. Tables III and IV demonstrate that the nonorosomucoid components of such mixtures in no way affected the immunochemical assay. On the other hand, by chemical estimation only 50 to 60 per cent of the added orosomucoid could be recovered due to its coprecipitation in perchloric acid with the albumin and gamma globulin.

TABLE III. EFFECT OF HUMAN SERUM ON DETERMINATION OF KNOWN QUANTITIES OF OROSOMUCOID

A SAMPLE CONTENT*	B KNOWN OROSO. CONTENT (MG./ML.)	C OROSO. CONTENT BY IMMUNOCHEMICAL ASSAY† (MG./ML.)
1. 1 ml. saline + 0.1 ml. 2.75 mg. oroso 7	2.50‡	2.56
2. 1 ml. 1 mg. oroso 8 + 0.1 ml. saline	0.91‡	0.80
3. 1 ml. 1 mg. oroso 8 + 0.1 ml. 2.75 mg. oroso 7	3.41‡	3.30
4. 1 ml. Z-948 + 0.1 ml. saline		0.74
5. 1 ml. Z-948 + 0.1 ml. 2.75 mg. oroso 7	3.24§	2.94
6. 1 ml. Z-901 + 0.1 ml. saline		1.25
7. 1 ml. Z-901 + 0.1 ml. 2.75 mg. oroso 7	3.75‖	3.78

*Mixtures of two purified orosomucoid preparations either with saline or with human serum. Z-948 and Z-901 are human serum samples.

†The nephelometric procedure was employed.

‡From biuret determinations of the two purified orosomucoid preparations. The biuret value of orosomucoid, employing human serum albumin as the biuret standard, was multiplied by the factor 1.47 to obtain the total weight of orosomucoid.

§The sum of row 1 (column B) + row 4 (column C).

‖The sum of row 1 (column B) + row 6 (column C).

TABLE IV. THE EFFECT OF ALBUMIN AND GAMMA GLOBULIN CONCENTRATION OF THE RECOVERY OF OROSOMUCOID AS ASSAYED BY THE SEROMUCOID TEST AND THE IMMUNOCHEMICAL TEST

SAMPLE CONTENT	MG. OROSOMUCOID PER ML. AS ASSAYED BY:	
	SEROMUCOID TEST*	IMMUNOCHEMICAL TEST†
A. 1 mg. human orosomucoid 7 per ml. 50 mg. Rwk Fr V (human) per ml. 25 mg. Fr II (human per ml. Buffered 0.9 per cent NaCl, pH 7.6	0.53	1.03
B. 1 mg. human orosomucoid 7 per ml. 25 mg. Rwk Fr V (human) per ml. 25 mg. Fr II (human) per ml. Buffered 0.9 per cent NaCl, pH 7.6	0.60	0.97
C. 1 mg. human orosomucoid 7 per ml. Buffered 0.9 per cent NaCl, pH 7.6	1.00	0.95

*Seromucoid hexose assay as described by Winzler[4] employing sample C as the standard for A and B.

†The Beckman spectrophotometric procedure was employed.

Serum Orosomucoid Levels Compared to Mucoprotein Levels.—Fig. 4 compares the serum orosomucoid levels of 74 nomal adults and 142 cancer patients.* The normal samples were drawn from healthy laboratory workers and from hospital blood donors The cancer samples were from patients with various malignancies, mainly in advanced inoperable stages Serial determinations have been performed on a number of these patients for months at a time, but for purposes of this paper only one value per patient is recorded In each case the value was the first one determined on the patient during the time span encompassed by receipt of samples Z 715 to Z 1500 The orosomucoid values in the normals ranged from 37 mg per cent to 145 mg per cent with a mean of 76 mg per cent and in the cancer group ranged from 68 mg per cent to 390 mg per cent with a mean of 177 mg per cent In spite of the wide range of values in the cancer sera, there was a striking correspondence between the increased mean of our series and those reported by various investigators for serum mucoprotein concentration [4 10 11 14] This is to be expected since orosomucoid is the principal component responsible for the chemical estimations

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*Since the results were tabulated, a considerable number of additional patients have been added to the series.

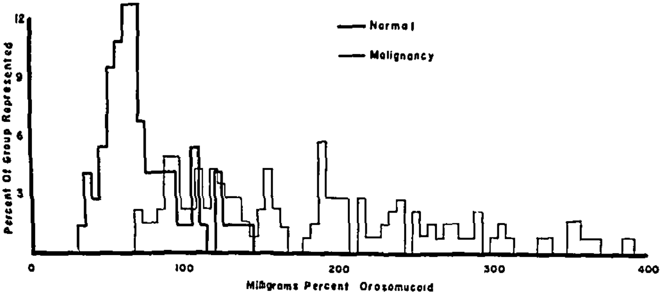

FIG 4—Distribution of serum orosomucoid levels in normal adults and patients with malignancy 74 normals compared to 142 patients

TABLE V COMPARISON OF MUCOPROTEIN CONTENT IN NORMAL AND CANCER SERUM BY VARIOUS ASSAY METHODS

	NORMAL	CANCER	RATIO $\frac{C}{N}$	REFERENCE
Immunologic orosomucoid test*	76 0 ± 2 99†	177 0 ± 6 49†	2 33	Present experiments
Polarographic filtrate test‡	1 0	2 4 ± 0 03	2 40	*Brdicka et al*[11]
Mucoprotein§	2 7 ± 0 05	6 1 ± 0 13	2 26	Winzler and Smyth[10]
Mucoprotein‖	136 5 ± 3 93	308 0 + 15 4	2 25	Lockey et al[14]

*Milligram per cent orosomucoid

†Standard error

‡Recorded as ratio $\frac{\text{mm wave length for cancer serum}}{\text{mm wave length for normal serum}}$

§Milligram per cent of tyrosine of the serum fraction soluble in perchloric acid but insoluble in phosphotungstic acid

‖Milligram per cent of mucoprotein that serum fraction soluble in sulfosalicyclic acid but insoluble in phosphotungstic acid

A comparison of values obtained by these different tests is presented in Table V Nine tuberculosis serum samples and 12 pregnancy samples were also analyzed for orosomucoid content There were elevated values in the tuberculosis group, but in the pregnancy group orosmucoid was at normal concentration This, too, is in accord with the results of chemical serum mucoprotein estimation. It is well to note that the range and nonspecificity in serum levels of orosomucoid and the chemical mucoprotein fractions are so great as to preclude their being of diagnostic value in cancer.

DISCUSSION

The current study represents an extension of our efforts to develop an inclusive system for the quantitation of human plasma proteins by the use of chicken antisera. Pertinent to this objective has been the observation that at 13 per cent NaCl far more potent reactions can be obtained with antiorosomucoid serum than at 8 per cent NaCl. To varying degrees this high salt effect is manifested by all the chicken antiserum-antigen systems studied so far. The weaker or more dilute an antiserum the greater is the difference in precipitation between 13 and 8 per cent. Consequently, antisera in small supply such as we have produced to several alpha-2 glycoproteins are now being stored and reacted at the higher salinity.

The current work further substantiates our previous observation[1] that certain antisera lacking complete specificity can be used to gather reliable data. For antiserums such as P-30 this may have been fortuitous in that the "unwanted" antibodies were directed to a protein whose human serum concentration appears to be very low.* However, our use of a calibrated pool of human serum as the assay standard may well have neutralized slight differences in reactivity between the various antiorosomucoid sera.

The mean serum concentration of orosomucoid in normals was found to be 76 mg. per cent. This value is lower than the 103 mg. per cent reported by Silberberg and associates[15] and the 119 mg. per cent reported in our preliminary studies.[22] The orosomucoid preparations used as the assay standards in the earlier work may have been less pure or more altered in specificity than the orosomucoid used to calibrate PCS, the routine standard of the current study. Our value of 76 mg. per cent is in accord with the data gathered for the seromucoid or serum mucoprotein fraction. De La Huerga and associates[23] have established by a turbidimetric assay that the mean value for the chemical fraction in normal serum is 79.4 mg. per cent. This value corresponds to mean values for other groups of normals obtained by biuret,[24] tryrosine,[10] and hexose[4, 10] methods.† Upon adjusting for the estimated 30 per cent loss during perchloric acid precipitation,[9] the total normal serum mucoprotein concentration can be considered 113 mg. per cent, 67 per cent of which is orosomucoid. These results agree with the observed electrophoretic heterogeniety of seromucoid[16] in which its major component (MP-1) possesses the physicochemical properties of orosomucoid.

The specific immunochemical measurement of orosomucoid has greater accuracy than the various serum mucoprotein assay methods. These latter have an inherent technical error in that the recovery of seromucoid is dependent on the concentration and type of proteins in the serum sample under assay.

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*This protein is concentrated in the Zn precipitable alpha-2 glycoprotein fraction characterized by Schmid.[3]

†De La Huerga and associates[23] discuss the correspondence of these various chemical methods of assaying the serum mucoprotein fraction.

Breakdown products of proteins can also occur in the chemical fraction after storage of serum Furthermore, the relative distribution of the components in seromucoid may vary according to the physiologic stimuli Mandel and associates[25] have pointed out the uncertainty this introduces into the evaluation of seromucoid determinations In diseases such as malignancy and tuberculosis, both orosomucoid and seromucoid increase in concentration over normal levels and show a comparable spread of values in randomly selected patients Yet the two measures may not necessarily parallel each other in individual patients subjected to various forms of treatment For example Bollet[26] has reported that in some patients cortisone administration caused an increase in the total protein of seromucoid and a concomitant relative decrease in the hexosamine of the fraction Our own work[2] indicates that in cancer the orosomucoid level is not directly affected per se by cortisone or a number of other therapeutic compounds, but is affected by a gross change in the clinical state of the patient, increasing with clinical deterioration and decreasing with clinical improvement That seromucoid and orosomucoid levels need not be closely correlated is also indirectly suggested on comparison of these measures to the presence or absence in human sera of C Reactive Protein (CRP) Shetlar and associates[28] have claimed a close correlation between the elevation of seromucoid concentration and the appearance of CRP On the other hand our data (unpublished) has shown a number of samples in which orosomucoid concentration was elevated and CRP absent The reverse was also found The possible differences in data gathered by the seromucoid and the immunochemical orosomucoid assay methods point to the need to analyze the constituents of serum in terms of discrete protein species The fluctuations in serum concentration of such proteins as orosomucoid may provide a more definitive characterization of the processes operative in disease

SUMMARY

The measurement of serum orosomucoid concentration by specific chicken antiorosomucoid serum has been demonstrated to be a practical laboratory method Rapid turbidimetric procedures for assay were worked out in which the reactions were carried out in 13 per cent NaCl The choice of this high salinity resulted from the observation that the effects of NaCl concentration were more pronounced in the chicken antiorosomucoid system than in other chicken antiserum systems The average serum orosomucoid level of normal adults was found to be 76 mg per cent ± 2 99 (s e) Marked increases of serum orosomucoid concentration were often found in malignancy and there was also a wider range of levels than in normal subjects The relationship of the orosomucoid determination to various serum mucoprotein assay methods was discussed

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THE CHEMICAL ESTIMATION OF EPINEPHRINE AND NOREPINEPHRINE IN HUMAN AND CANINE PLASMA

II. A Critique of the Trihydroxyindole Method

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INTRODUCTION

THE ethylenediamine condensation method of Weil-Malherbe and Bone[1] and its modifications have been extensively used to estimate the concentrations of epinephrine (E) and norepinephrine (N) in plasma, blood, tissues, and urine. Recently, it has been reported that this method is not sufficiently specific for these purposes.[2, 3]

The trihydroxyindole method of Lund[4] is more specific, but has been found to lack sufficient sensitivity to detect the extremely low concentrations of E and N *which normally occur in human and canine plasma.*[4-6]

In the work to be reported, means of increasing both the sensitivity and specificity of the trihydroxyindole method have been sought. It has been found (1) that the quantities of E and N present in a mixture can be estimated fluorometrically rather than by differential oxidation,[4] thereby reducing the volume of plasma needed for analysis by one third, (2) that the fluorescence produced by E and N is increased while that of certain interfering substances is reduced by adjusting the pH of the reaction products, and (3) that the precision of the determinations can be improved by a new method of preparing blanks. With these modifications 0.001 μg E or 0.002 to 0.003 μg N can be detected in plasma eluates. Satisfactory recovery of these quantities of E and N is demonstrated below, and the concentrations of these substances occurring in normal human plasma are estimated.

METHODS

Blood samples are collected in syringes moistened with heparin and are iced immediately. The plasma is separated as soon as possible by centrifuging the blood at 750 × g for 20 minutes. Chromatography of the plasma is completed within 3 hours after the blood is drawn.

A. *Alumina Column Chromatography.*—The columns used consist of glass tubes 25 cm. in length with an upper portion 5 cm. long and 0.8 cm. in diameter, a bulbous central part of 30 ml. capacity, and a lower portion 0.6 cm. in inside diameter and 10 cm. long. The bottom of the tube is crimped slightly to hold a glass wool plug which supports the alumina. With

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the column half-filled with distilled water and the glass wool plug in position, 1.0 Gm. of alumina* is poured into the top of the column and allowed to settle. Trapped air is removed from the alumina by agitation with a glass rod introduced through the top of the column before settling is complete. The supernatant water is forced through the column, using air pressure, until its level is just above that of the alumina. The alumina is washed with 5 ml. of glass-distilled water, and is then ready for use.

The plasma pH is adjusted to 8.0 ± 0.3 by swirling it in a beaker, causing loss of carbon dioxide and increase in pH, after which it is poured onto the alumina column. The plasma is forced through at an even rate (1 to 2 ml./min.) by suitable adjustments of air pressure. When the plasma level has reached that of the top of the alumina, 5 ml. 0.2 N sodium acetate followed by 5 ml. glass-distilled water is forced through the column. Columns which have been allowed to run dry at any point before elution are discarded. The alumina is eluted with 6 ml. of 0.3 N acetic acid. Eluates are collected in glass-stoppered tubes, in which they may be frozen and stored for several weeks before analysis, without significant loss of E or N.

B. *Analysis of Eluates.*—Merck reagent grade chemicals are used throughout, except as noted. The pH of each eluate fraction (2.0 to 3.0 ml.) to be analyzed is adjusted to 6 by drop-wise addition of 1 N sodium hydroxide after the addition of 0.04 per cent bromthymol blue indicator (Harleco), and 1.0 ml. 1 N sodium acetate buffer (pH 7.6 to 8.0). The correct end point is indicated by the appearance of a persistent light-green color. Samples which become more alkaline, as indicated by the appearance of a persistent blue color, should be discarded.

An eluate fraction is then run as a *sample* by adding 0.1 ml. of 0.25 per cent potassium ferricyanide solution, mixing, and allowing the mixture to stand 5 minutes. After this 0.10 ml. of 2.0 per cent ascorbic acid is added, followed immediately by 0.9 ml. of 20 per cent sodium hydroxide. The mixture can then be diluted to standard volume (6 to 7 ml.) and read in a fluorometer, but for reasons to be discussed later, we routinely convert the pH of the mixture to approximately 5 by the addition of 2.0 ml. of 5 N acetic acid 4 minutes after the addition of ascorbic acid and before diluting to final volume.

A *blank* is prepared from another equal fraction of the eluate after titrating it to pH 6. Ascorbic acid, followed by ferricyanide and sodium hydroxide are added in the same amounts used to prepare samples. Four minutes later the pH is brought to 5 by adding 5 N acetic acid as in the samples.

Samples and blanks are read in a Farrand photofluorometer at equal times (7 and 10 minutes) after the addition of sodium hydroxide. Light of two wave lengths (391 to 408 and 436 mμ) is used to excite the solution; the emission is read at 500 mμ. The filter combinations used are: (exciting) 400 mμ Farrand interference plus Wratten No. 35, and 436 mμ Farrand interference plus Wratten No. 35; and (emitting) 500 mμ Farrand interference plus Wratten No. 57.

Standard additions of E and N (Sterling-Winthrop: 0.01 to 0.05 μg of base) are made to plasma eluates or to "blank" eluates prepared by running reagents only through alumina columns and eluting with acetic acid as described above. Additions made to reagents which have *not* been exposed to alumina are unacceptable for analytic purposes because the fluorescence observed per microgram of E and N and the ratios of fluorescence observed using the 400 and 436 mμ primary filters are modified by the presence of the acetic acid eluate of alumina.

Calculations of E and N concentration are performed by comparing the fluorescence obtained per microgram of added E and N with the net fluorescence (sample reading minus blank reading) observed in the unknown. This procedure yields simultaneous equations which can be solved algebraically:

Equation 1: $F\ 400 = mE + nN$

Equation 2: $F\ 436 = oE + pN$, where F_{400} and F_{436} are the net (sample minus blank) number of galvanometer divisions observed when the sample is excited by light of the two wave lengths, m, n, o, and p are the number of galvanometer divisions deflection per microgram of E or N added to the standards, and E and N are the quantities of E and N present in the unknown mixture.

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*Woelm, nonalkaline, Grade I, Alupharm Chemical Co., New Orleans, La.

This method of analysis requires, for correctness, that the fluorescence of a mixture equal the sum of that produced separately by the fluorescent substances present, and that fluorescence be proportional to concentration. Both requirements are met in the concentration range tested.

TABLE I FLUORESCENCE OF HYDROXYPHENYL COMPOUNDS

SUBSTANCE	RING*	SIDE CHAIN	FILTER $(M\mu)$	RELATIVE FLUORESCENCE (%)	
				pH_5	pH_{13}
l Epinephrine	R_1-	CHOHCH NH	400	100.0	85.0
		| CH_3	436	100.0	76.0
DL Isopropylnorepinephrine	R_1-	CHOHCH NH	400	72.0	--
		| $CH(CH_3)_2$	436	75.0	--
l Norepinephrine	R_1	CHOHCH NH_2	400	85.0	71.0
			436	37.0	27.0
Threo DL 3,4 dihydroxyphenyl serine	R_1	$CHOHCHNH_2$	400	0.19	0.08
		| COOH	436	0.08	0.08
Erythro DL 3,4 dihydroxyphenyl serine	R_1	CHOHCHNH	400	0.03	0.01
		| COOH	436	0.01	0.001
DL Dopa	R_1-	CH $CHNH_2$	400	0.031	5.0
		| COOH	436	0.002	0.05
Dopamine	R_1	CH_2CH NH_2	400 436	0.13 0.04	0.14 0.04
3,4 Dihydroxyphenylacetic acid	R_1-	CH_2COOH	400 436	0.004 0.002	0.014 (-)0.002
3,4 Dihydroxymandelic acid	R_1	CHOHCOOH	400 436	(-)0.001 (-)0.001	-- -
5 Hydroxytryptamine (serotonin)	R_2-	3 CH CH NH_2	400 436	0.022 0.027	0.11 0.26

*R_1 = 3 4 dihydroxyphenyl R_2 = 5 hydroxyindole

RESULTS

1 *Fluorescence of Hydroxyphenyl Compounds* *—The fluorescence of certain compounds which occur biologically and which might interfere with the method is reported in Table I. All results are expressed in terms of the base or acid, and are referred to the fluorescence of adrenolutine (fluorescent product of epinephrine) at pH 5. Samples to be read at pH 5 were acidified by the addition of 2.0 ml 5 N acetic acid 4 minutes after the addition of 20 per cent

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*We wish to thank Dr M L Tainter Sterling-Winthrop Research Institute for supplying epinephrine norepinephrine and isopropylnorepinephrine Dr Richard Dahlbom Astra Ltd for supplying 3 4 dihydroxyphenylacetic acid Dr M D Armstrong University of Utah for supplying 3 4 dihydroxymandelic acid and Dr W G Clark Veterans Administration Center Los Angeles 25 for supplying the dihydroxyphenylserines. Dopa dopamine and serotonin were obtained from the California Foundation for Biochemical Research.

sodium hydroxide. Blanks were prepared in an analogous manner. Each blank eluate contained an addition equal to that of the corresponding sample eluate. Additions of E, N, and isopropylnorepinephrine ranged between 0.02 and 0.10 μg; additions of the other substances varied from 50 to 100 μg.

Conversion of the sample pH from 13 to 5 reduced the fluorescence of dopa, 3,4-dihydroxyphenylacetic acid, and 5-hydroxytryptamine, but increased that of E and N. This procedure also reduced the blank readings. However, adrenolutine and noradrenolutine are unstable at pH 5, so that all readings must be made at a standard time after the addition of 20 per cent sodium hydroxide if the final pH is to be acid. In all subsequent analyses the pH has been adjusted to 5, the samples read at 7 and 10 minutes after the addition of sodium hydroxide, and the results averaged. This applies whether an eluate fraction is treated as a sample or as a blank.

2. *Error of Fluorometric Separation of E and N.*—Amounts ranging from 0.002 to 0.02 μg E and 0.005 to 0.1 μg N were added to blank column eluates, analyzed by the method described, and compared with eluates to which nothing had been added. The results are shown in Table II. Proportions ranging from 1:1 to 10:1 and amounts ranging from 0.1 to 0.002 μg could be separated fluorometrically with an average error of 4.6 per cent (range 0 to 14 per cent).

TABLE II. FLUOROMETRIC SEPARATION OF E AND N

μG ADDED		μG RECOVERED		PER CENT	
E	N	E	N	E	N
0.022	0.044	0.022	0.042	100	96
0.022	0.044	0.022	0.044	100	100
0.011	0.099	0.011	0.090	100	91
0.0055	0.0055	0.0063	0.0058	114	105
0.0055	0.0555	0.0054	0.0579	98	104
0.0022	0.0022	0.0020	0.0020	91	91

3. *Recovery of E and N by Alumina Column Chromatography.*—Amounts of E ranging from 0.12 to 0.002 μg were added to twenty-four 15 ml. samples of human and canine plasma which were then chromatographed on alumina. These eluates were analyzed and compared with others obtained from plasma to which nothing had been added, and the recovery calculated. The mean recovery was 94.2 per cent, with standard deviations of 10.5 per cent at the upper half of the range and 26.5 per cent at the lower. Seventeen additions of N ranging from 0.1 to 0.006 μg were recovered to the extent of 86.3 per cent with a standard deviation of 6.7 per cent at the upper half of the range and 20.2 per cent at the lower. There was no consistent difference between recoveries from canine and human plasma. The increased variation in apparent recovery observed when small additions are made to plasma is of the same order as the variation in replicates of a single sample, and thus probably represents the "background" variation in the analytic method which becomes apparent when low concentrations of E and N are analyzed rather than variation in the recovery from alumina.

4. *Evaluation of Blanks.*—Arterial plasma from 4 normal human volunteers and 2 dogs was allowed to stand at refrigerator temperature (4 to 7° C.) for 4 to 5 weeks, and then chromatographed on alumina columns. By this time more than 85 per cent of the N added to small fractions of the fresh plasma had disappeared.

Eluates of aged plasma were prepared as blanks by two procedures different from that described in the Method. Oxidation of the eluate at pH 6, followed by addition of NaOH, with addition of ascorbic acid 4 minutes later resulted in erratic blanks whose fluorescence occasionally exceeded that of other portions of the same eluate which had been treated as samples. Blanks prepared by omitting the oxidation step ($K_3Fe[CN]_6$ not added) were considerably lower than samples prepared from the same eluates.

For these reasons the technique outlined in the Method was used routinely to prepare blanks. Fluorescence was measured at pH 5. With this technique the difference between eluates of aged plasma treated as blanks and samples was small. Seven aged plasma samples averaged 0.023 μg/L. apparent E and 0.046 μg/L. apparent N. In eluates prepared from reagents only, there was no apparent E or N.

TABLE III. BLANK FLUORESCENCE AS PER CENT OF TOTAL

SUBSTANCE	AMOUNT ADDED μG	$100 \times \left[\frac{\text{FLUORESCENCE OBSERVED IN BLANK WITH ADDITION}}{\text{FLUORESCENCE FROM SAME ADDITION TREATED AS A SAMPLE}}\right]$
E	0.04	17
	0.04	14
	0.04	13
	0.04	24
	0.04	13
	0.04	29
	0.04	20
	0.02	6
	0.04	8
	0.04	21
	0.04	9
	0.02	17
	0.04	24
	0.04	25
		Average 17.1 ± 7.0 (s.d.)
N	0.04	2
	0.04	4
	0.04	4
	0.08	2
		Average 3.0 ± 1.1 (s.d.)

The blank values obtained by the method described are proportional to the E and N content of the sample (Table III). The explanation of this finding is that a small part of the E and N is changed, during adjustment of the pH to 6 with sodium hydroxide, to substances which subsequently form adrenolutine and noradrenolutine at high pH. The amount of noradrenolutine "preformed" in the blanks is negligible (less than 5 per cent); that of adrenolutine can be reduced to about 15 to 20 per cent by careful titration with agitation. Too rapid titration or insufficiently thorough mixing during titration may yield blank values up to 40 per cent of the E present.

The over-all recovery of E or N equals the recovery from alumina multiplied by the recovery during the titration and oxidation steps. For E this is 100 × 0.94 × 0.83 ≈ 78 per cent, and for N is 100 × 0.86 × 0.97 ≈ 83 per cent. The apparent plasma concentrations of E and N reported below have not been corrected for these losses.

5. *Standard Deviation of a Single Determination.*—Four 30 ml. plasma samples were divided in half, and each fraction was separately chromatographed on an alumina column. Half of each eluate was treated as a sample, the other half as a blank. The standard deviation of a single determination was calculated as s.d. $= [\Sigma d^2/_{2n}]^{1/2}$, where d is the difference in estimated concentration between the duplicate analyses of each plasma sample, and n is the number of duplicates used in the calculation. The results were: s.d. of E determination = 0.042 μg/L. and of N = 0.13 μg/L.

6. *Normal Plasma Concentrations of E and N in Man.*—Thirty-milliliter blood samples were withdrawn from the brachial arteries or antecubital veins of 12 normal adults. In 7 cases the arterial and venous samples were withdrawn simultaneously. The subjects rested in the supine position during sampling and for 10 minutes before. All were hospital personnel (physicians, medical students, or nurses), and all appeared free from apprehension. The data are shown in Table IV. All concentrations are in terms of the bases, and are uncorrected for losses in recovery which average approximately twenty per cent (paragraph 4).

TABLE IV. NORMAL PLASMA E AND N CONCENTRATIONS ESTIMATED BY THE TRIHYDROXYINDOLE METHOD

SUBJECTS			SOURCE OF SAMPLE				DIFFERENCE	
			BRACHIAL ARTERY		ANTECUBITAL VEIN		A-V	
NO.	AGE	SEX	E (μg/L.)	N	E (μg/L.)	N	E (μg/L.)	N
1	25	F	0.00	0.25	0.05	0.34	(-)0.05	(-)0.09
2	26	M	0.13	0.18	(-)0.08	0.25	0.21	(-)0.07
3	23	M	(-)0.02	(-)0.08	0.06	0.11	(-)0.08	(-)0.19
4	23	F	0.25	0.25	0.00	0.46	0.25	(-)0.21
5	27	F	0.00	0.35	(-)0.07	0.43	0.07	(-)0.08
6	23	M	0.18	0.18	0.18	0.34	0.00	(-)0.16
7	26	F	0.18	0.26	0.02	0.58	0.16	(-)0.32
8	34	M	--	--	0.00	0.16	--	--
9	24	M	0.09	0.23	--	--	--	--
10	30	M	--	--	(-)0.01	0.19	--	--
11	28	M	--	--	0.01	0.48	--	--
12	24	M	--	--	(-)0.01	0.29	--	--
Average			0.10 ± 0.10 s.d.	0.20 ± 0.12 s.d.	0.01 ± 0.07 s.d.	0.34 ± 0.15 s.d.		

The levels of E in arterial plasma averaged 0.10 μg/L. and this concentration was significantly greater than zero ($p < 0.05$). The concentration of E in venous plasma averaged 0.01 μg/L. and was insignificantly different from zero. The arteriovenous concentration difference of E was positive or zero in 5 of the 7 subjects from whom simultaneous samples were drawn, but a probably significant difference (0.16 μg/L or more) was observed in only 3 cases, and the difference was statistically significant for neither the group as a whole nor for the A-V group.

The levels of N in venous plasma consistently exceeded those in arterial plasma. The difference in concentration averaged 0.14 μg/L in the group as a whole and 0.16 μg/L in the A-V group. The difference is statistically significant ($p < 0.01$) for the A-V group, but not for the group as a whole.

DISCUSSION

A serious limitation of chemical methods is that they do not establish the biologic activity of the substances estimated. Unfortunately, the concentrations of E and N present in human plasma are too low to permit estimation by the biologic assay methods currently employed. Holzbauer and Vogt[7] have shown the venous plasma of a single subject to contain less E and N than the minimum concentrations detectable by a sensitive biologic method, i.e., less than 0.06 μg/L E and 1.0 μg/L N. By the present (trihydroxyindole) method the concentration of E in venous plasma is insignificantly different from zero while that of N is less than 0.6 μg/L. By the ethylenediamine method concentrations of E range from 0.1 to 1.3 μg/L and of N from 2.1 to 5.2 μg/L in normal human venous plasma[1-3], the reason for the variation in the results obtained is obscure. In addition to E and N, the ethylenediamine method is sensitive to the presence of dopamine, as well as to some of its supposed precursors and metabolites,[3] and probably to metabolites of N as well.

The trihydroxyindole method theoretically detects only 3,4-dihydroxyphenyl compounds with a two-carbon side chain, a terminal nitrogen, and an hydroxyl group substituted on the β carbon atom.[8] Thus it should be insensitive to any of the hydroxyphenyl compounds, other than E and N, which are presently known to occur to an important extent in man or dog. Isopropyl norepinephrine, which could seriously interfere with the determination of E by the present method, may occur in the human adrenal medulla, but only in trace amounts.[9] Dopamine, which occurs in human urine, has been reported by von Euler and Floding[10] to produce fluorescence equal to 3 per cent of that of N by the trihydroxyindole method. With the present technique, the fluorescence of dopamine is approximately 0.1 per cent of that of N (Table I). A partial explanation of the difference lies in our exciting the samples at 400 mμ in addition to 436 mμ, and in reading the samples at pH 5, both of which increase sensitivity for N without appreciably modifying that for dopamine. However, a large part of the greater specificity of the present method is unexplained, and may be attributable to the optical system employed. It may also be pointed out that the fluorescences of serotonin and dopa at pH 13 are unduly high, and that measuring fluorescence at pH 5 causes a tenfold and a hundred-fold decrease, respectively, in sensitivity for these substances.

The development of valid "blanks" for methods measuring endogenous substances is difficult. In the case where the substance analyzed is not normally present in the body blanks consist simply of analyses made before or at a suitable interval after administration of the foreign substance. In the present case, however, a theoretically ideal solution, in which the fluorescence developed by alkalinizing adrenochrome and noradrenochrome[4] is permitted to fall to zero by withholding ascorbic acid, appears inacceptable since the "blanks" frequently give higher readings than the unknowns. Obviously some aspect of the blanking technique causes one or more constituents of the eluates to emit or transmit more light than in the samples, and certainly the constituents concerned cannot be E or N. A large proportion of the "fluorescence" read by any fluorometer appears to result from reflected light (an empty cuvette gives a high reading), suggesting that the measurements must be a mixture of fluorometry, colorimetry, and nephelometry. In this circumstance the selection of a "blank" is to some extent arbitrary, since different solutions, none of which is fluorescent, may produce different readings in a fluorometer. We have retained the present blanking technique because it appears to be the only alternative to the original method[4] in which all constituents of the final mixture are present equally in samples and blanks. In the blanks, oxidation of E and N is prevented by the addition of ascorbic acid prior to the addition of ferricyanide, and consequently fluorescence does not develop when the mixture is made alkaline. A blanking method omitting ferricyanide entirely should achieve the same purpose, but we have rejected it because readings obtained by this method are consistently lower than when ferricyanide is added to the blanks. This is true even in plasma which has been blanked by aging, indicating that ferricyanide contributes to the "fluorescence" measured independently of its action on E or N.

The method described may be sufficiently sensitive to detect evidence of the resting secretion of the adrenal medullae, i.e., the difference between normal arterial plasma E concentrations and zero. This difference amounts to approximately 0.1 μg/L. which has been calculated previously[11] to result from the secretion of 0.1 to 0.2 μg/min. E from the adrenal medullae.

The significantly greater antecubital venous than arterial concentration of N presumably reflects the secretion of this substance from sympathetic nerves supplying the forearm. Studies are planned to determine whether a significant arteriovenous concentration difference also exists in organs such as brain, heart, and liver.

SUMMARY

1. A fluorometric method has been described which is capable of detecting as little as 1 Mμg of epinephrine and 2 to 3 Mμg of norepinephrine.

2. Of a series of biologically occurring substances of similar chemical structure, only isopropylnoradrenaline interfered with the detection of epinephrine or norepinephrine by this method. Isopropylnoradrenaline resembled epinephrine, but could be distinguished from norepinephrine.

3 The concentrations of epinephrine and norepinephrine in normal human arterial plasma were estimated as 0 10 + 0 10 (s d) and 0 20 ± 0 12 (s d) micro grams per liter, respectively

4 The concentration of epinephrine in antecubital venous plasma was lower, and that of norepinephrine higher, than in arterial plasma, the difference being statistically significant for norepinephrine but not for epinephrine

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THE EFFECT OF PRIOR CONTACT BETWEEN ACID CITRATE DEXTROSE AND SODIUM RADIOCHROMATE SOLUTIONS ON THE EFFICIENCY WITH WHICH Cr^{51} LABELS RED CELLS

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INTRODUCTION

SINCE Gray and Sterling[1] showed that human red cells contained about 20 μg per 100 ml. of chromium, Cr^{51}-labeled erythrocytes have been used to measure the body red cell mass and the in vivo red cell survival time. During experiments in this department to determine the optimum conditions for the tagging of red cells with Cr^{51} it was accidentally observed that the admixture of sodium chromate ($Na_2Cr^{51}O_4$) with an acid citrate dextrose (A.C.D.) solution before the addition of red cells (not after, as is the usual practice) led to comparatively poor labeling.

As such a finding appeared to preclude the preparation of stock mixtures of A.C.D. and $Na_2Cr^{51}O_4$ solutions in anticipation of their use, a series of experiments was designed to elucidate the reaction involved. While this work was in progress Cooper and Owen[2] reported impaired Cr^{51} labeling of erythrocytes when $Na_2Cr^{51}O_4$ solution was mixed with either A.C.D. solution or citrated plasma prior to the addition of blood. In explanation they postulated that "plasmatic reducing substances" converted the chromate to the chromic ion, which does not penetrate the red cell membrane but becomes bound to the plasma proteins.

Our investigations confirm that, under certain circumstances, reduction of the chromate ion does occur. They suggest that a chromic-citrate complex forms so that the chromic ion does not become bound to the plasma protein. The results appear to be of sufficient theoretical interest, as well as of enough practical importance to justify their publication.

METHODS

A preliminary experiment established that, at normal hematocrit values, more efficient labeling of red cells occurred in A.C.D. solution (1 part to 4 parts whole blood) than in saline suspension of red cells or when heparin was used as the *anticoagulant* (*Fig.* 1).

At lower hematocrit values, however, poorer labeling occurred in A.C.D. solution than in saline (Fig. 2).

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With this knowledge the experiments were designed using A C D. anticoagulant and blood from patients with normal hematocrit values Eight milliliters of whole blood or saline suspension of washed red cells were added to 2 ml. of the A C D. solution, or of the other test fluids, with which 5 μc of Cr^{51} had been mixed for varying periods of time The mixtures were then incubated at 37° C for 15 minutes Thereafter, the red cells were separated by centrifugation for 5 minutes at 2,000 r p m at a radius of 25 cm and were washed once in normal saline. The red cells were hemolyzed by saponin before their radio activity was assayed Radioactivity was measured in an EKCO liquid scintillation counter, connected with an EKCO autoscaler The sensitivity of the apparatus for Cr^{51} was approximately 14,000 c p m. per microcurie per 10 ml with a background of 75 c p m The uptake of Cr^{51} by the red cells was recorded by expressing the radioactivity of the red cells as a percentage of the total radioactivity of the cells and washings

For the sake of brevity the essential details of the individual experiments are taken along with their results

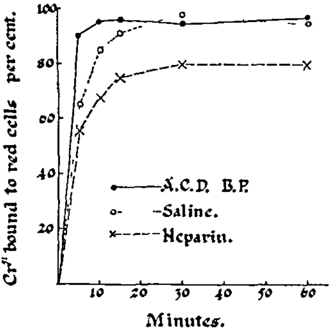

Fig 1

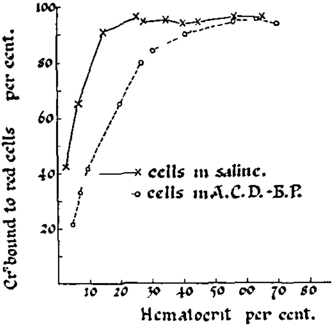

Fig 2

Fig 1 —Comparison of efficiency of labeling of red cells with Cr^{51} in A C D (B P), saline, and heparin

Fig. 2 —Comparison of efficiency of labeling of red cells with Cr^{51} at different hematocrit values

RESULTS

1. *The Effect of Prior Contact Between $Na_2Cr^{51}O_4$ and A.C.D. Solution.*—When $Na_2Cr^{51}O_4$ was left in contact with A.C.D solution before the addition of blood, the labeling of the red cells was impaired. Fig 3 demonstrates that the longer the contact the greater the deterioration in labeling efficiency. It also shows that deterioration is more rapid with A.C.D. (B.T.S *) solution than with A C D. (B P.†) solution

2. *The Effect of Variation in Concentration of Sodium Citrate.*—Each of 5 solutions of disodium citrate, the citrate concentration such that it varied from 1 2 to 2.68 per cent after the addition of $Na_2Cr^{51}O_4$, was allowed to react for

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*A.C D (B.T S.) (Blood Transfusion Service) pH at 5 1 sodium acid citrate (2 per cent) and dextrose (3 per cent).

This solution is sterilized by autoclaving

†A C D (B P) (British Pharmacopeal Formula) pH at 5 1 trisodium citrate dihydrate (1 29 per cent), citric acid monohydrate (0 46 per cent), and dextrose (2 5 per cent)

These proportions of trisodium citrate and citric acid yield in solution disodium hydrogen citrate The citrate and dextrose solutions are prepared and are autoclaved separately The final mixture is resterilized by Seitz filtration

1 hour. In all of these solutions, especially the more concentrated, labeling was impaired compared with controls in which the Cr^{51} was added after the blood (Table I).

3. *The Effect of Citrate Solution of Varying pH.*—Equimolar solutions (0.0847 M) of mono-, di-, and trisodium citrate of pH 4, 5.1, and 8, respectively, were allowed to react for 1 hour with the $Na_2Cr^{51}O_4$ solution. Labeling of the red cells was impaired in the more acid solutions (Table II).

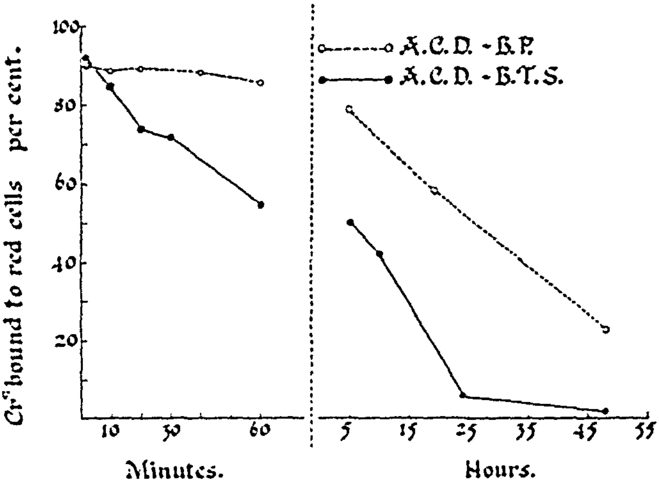

Fig. 3.—The effect of prior contact between $Na_2Cr^{51}O_4$ and A.C.D. solutions on the efficiency of labeling of red cells with Cr^{51}.

TABLE I. THE EFFECT OF VARIATION IN CONCENTRATION OF SODIUM CITRATE ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

CONCENTRATION OF CITRATE (PER CENT)	PER CENT Cr^{51} BOUND TO RED CELLS	
	PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND CITRATE	
	1 HOUR	NIL
1.2	77	96
1.6	79	96
2.0	72	95
2.4	65	95
2.68	49	93

TABLE II. THE EFFECT OF VARIATION IN pH OF CITRATE SOLUTIONS ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

SOLUTION	pH	PER CENT Cr^{51} BOUND TO RED CELLS	
		PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND CITRATE	
		1 HOUR	NIL
Monosodium citrate	4.0	60	87
Disodium citrate	5.1	76	89
Trisodium citrate	8.0	91	93

4. *The Effect of Variation in Concentration of Dextrose.*—Five solutions of dextrose (unbuffered), of which the final concentration after the addition of $Na_2Cr^{51}O_4$ solution was 1.2 to 2.68 per cent, were allowed to react for 1 hour. They produced slight but consistent impairment of labeling compared with simultaneous controls (Table III).

5 *The Effect of Dextrose Solutions of Varying pH*.—Solutions of dextrose of 2.4 per cent final concentration and pH of 2, 6, and 10 were allowed to react with the $Na_2Cr^{51}O_4$ solution for 19 hours. The pH of the mixture was adjusted to approximately 7 before the addition of red cells. Control experiments for each pH were done with saline in place of dextrose. The dextrose solutions diminished the ability of the Cr^{51} to label red cells. This effect was most pronounced in acid solution. Less pronounced loss of labeling was observed in the saline controls (Table IV).

TABLE III. THE EFFECT OF VARIATION IN CONCENTRATION OF DEXTROSE ON EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}

CONCENTRATION OF DEXTROSE (PER CENT)	PER CENT OF Cr^{51} BOUND TO RED CELLS	
	PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND DEXTROSE	
	1 HOUR	NIL
1.2	87	92
1.6	87	92
2.0	87	93
2.4	86	91
2.68	84	90

TABLE IV. THE EFFECT OF VARIATION IN pH OF DEXTROSE SOLUTIONS ON THE EFFICIENCY OF LABELING RED CELLS WITH Cr^{51}. PRIOR CONTACT BETWEEN $Na_2Cr^{51}O_4$ AND DEXTROSE OF 19 HOURS

pH OF DEXTROSE SOLUTION	PER CENT OF Cr^{51} BOUND TO RED CELLS	
	DEXTROSE	SALINE CONTROL
2	0	37
6	21	91
10	65	81

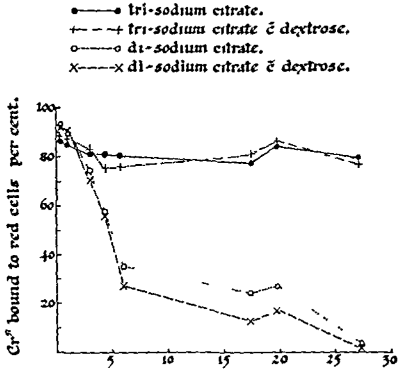

Fig 4.—The effect of prior contact between $Na_2Cr^{51}O_4$ and citrate solutions of pH 5.1 and 8 with and without glucose on the efficiency of labeling of red cells with Cr^{51}

6 *The Effect of Citrate Solutions With and Without Glucose*.—Solutions of sodium citrate of pH 5.1 and 8, respectively, were used, both with and without

3 per cent dextrose. When the $Na_2Cr^{51}O_4$ was left in contact with these solutions, the ability of the Cr^{51} to label red cells was impaired especially at the lower pH (Fig. 4). The longer the exposure the more noticeable was the effect which was probably also more obvious in the presence of dextrose.

7. *The Effect of Light on the Reaction Between the $Na_2Cr^{51}O_4$ and A.C.D. Solutions.*—As chromates have photosensitive properties, it was thought worth while to investigate the effect of light on the reaction between $Na_2Cr^{51}O_4$ and A.C.D. solution.

$Na_2Cr^{51}O_4$ was added to A.C.D. (B.P.) solution and part of the mixture kept in the dark while the remainder was exposed to the light of a 100 Watt tungsten lamp at a distance of 6 inches. The temperature of both solutions was controlled at 18° C. by means of a cirulating water bath. Assays of the labeling power of these mixtures at intervals showed that in the dark there was little impairment in the first 4 hours but in the light rapid loss occurred from the outset (Fig. 5).

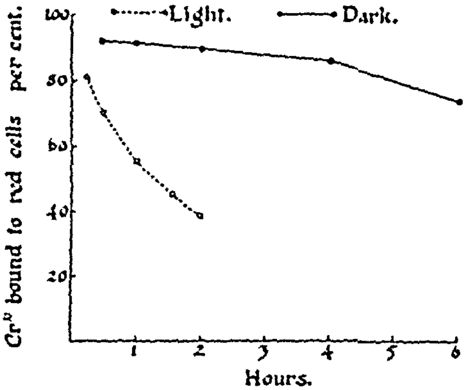

Fig. 5.—The effect of light on the reaction between $Na_2Cr^{51}O_4$ and A.C.D. solutions.

8. *The Effect of Autoclaving A.C.D. Solution.*—When A.C.D. solution was heated to 100° C. for 4 hours a yellow discoloration developed, indicative of early caramelization of the dextrose. The labeling power of $Na_2Cr^{51}O_4$ was impaired very rapidly when mixed with these solutions. (Table V).

Table V. The Effect of Autoclaving A.C.D. Solutions on the Efficiency of Labeling Red Cells With Cr^{51}

Duration of contact between $Na_2Cr^{51}O_4$ and A.C.D.	Per cent of Cr^{51} bound to red cells			
	A.C.D. (B.P.)		A.C.D. (B.T.S.)	
	Autoclaved	Not autoclaved	Autoclaved	Not autoclaved
30 min.	3	84	2	86
60 min.	2	81	2	88

9. *Attempts to Identify the Degradation Products of Sodium Chromate Formed in the Preceding Reaction.*—Evidence for the reduction of the chromate ion to the chromic ion was found. $Na_2Cr^{51}O_4$ solution was exposed to the action of 3 per cent dextrose in N/10 HCl, both in the light and in the dark

for 4 hours. Any chromic ions formed by reduction from chromate were then precipitated by ammonia. Carrier chromic ions were provided to facilitate handling of the precipitate and carrier chromate to minimize the loss of unreduced chromate due to adsorption by the fine precipitate. The precipitate was separated and redissolved in HCl so that its radioactivity could be estimated. The precipitation, along with the carrier chromic ion, of a proportion of the radioactivity, which increased with the passage of time and on exposure to light, indicated that, under the conditions of this experiment, chromate was reduced by dextrose (Table VI).

TABLE VI. PERCENTAGE OF Cr^{51} PRECIPITATED WITH CHROMIC IONS AFTER ADMIXTURE OF $Na\,Cr^{51}O_4$ WITH 3 PER CENT DEXTROSE IN N/10 HCL

DURATION OF CONTACT BETWEEN $Na\,Cr\,O_4$ AND 3 PER CENT DEXTROSE IN N/10 HCL	SOLUTION EXPOSED IN	
	LIGHT	DARK
1 Hour	38	14
4 Hours	59	31

The formation of complexes between chromic ions and citrate precluded the use of this method to study the reaction between $Na\,Cr^{51}O_4$ and citrate solutions or for that matter the use of the ion exchange resin technique.

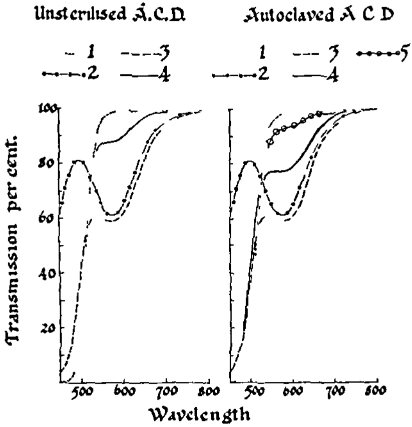

Fig. 6.—The effect of autoclaving A.C.D. (B.P.) solution on the rate at which it reacts with 1 per cent K_2CrO_4 solution. Curves 1, 2, and 3 are the transmission spectra of freshly prepared mixtures of A.C.D. (B.P.) solution with 1 per cent K_2CrO_4, 1 per cent $Cr_2(SO_4)_3$, and 0.5 per cent K_2CrO_4 plus 0.5 per cent $Cr_2(SO_4)_3$. Curve 4 is the transmission spectrum of 1 per cent K_2CrO_4 solution mixed with A.C.D. (B.P.) solution after 11 days' exposure to light. Curve 5 is the same for a similar mixture kept in the dark for 11 days.

Instead a spectrophotometric analysis was made of mixtures of 1 per cent $K\,CrO_4$ with acid dextrose (N/10 HCl), A.C.D. (B.P.), citric acid, monosodium citrate, and disodium citrate solutions. The same method was used to study the reaction between K_4CrO_2 and A.C.D. (B.P.) solution, and between K_2CrO_4 and 3 per cent dextrose in 2 per cent acid citrate solution, before and after autoclaving.

Solutions which contain chromic ions have an absorption band maximal between 570 and 580 mμ. An absorption band in this position developed in the test solutions except in the mixtures with disodium citrate where little or no reaction was observed. The absorption band became more intense with time, the change being more rapid in solutions exposed to light. When solutions containing dextrose were autoclaved, the appearance of the absorption band was more rapid than in identical solutions which were not autoclaved (Fig. 6).

DISCUSSION

With the standard technique used in our department excellent labeling of red cells occurs. Four parts of whole blood are mixed with 1 part of A.C.D. solution and $Na_2Cr^{51}O_4$ is then added. The mixture is incubated at 37° C. for 15 minutes. Provided that the hematocrit is 40 per cent or more approximately 90 per cent of the Cr^{51} is taken up by the red cells. In saline suspensions of red cells, even when the hematocrit of the mixture is as low as 20 per cent, equally good labeling occurs. Cooper and Owen[2] reported similar efficient labeling in A.C.D. solution with blood of normal hematocrit values. They also noted diminution of the labeling efficiency as the hematocrit fell. They observed that labeling with Cr^{51} never reaches 100 per cent, a finding which they attributed to the conversion, by "plasmatic reducing substances" of a small amount of the chromate ion to the cationic chromic state, in which they considered it would become bound to the plasma protein.

The details of their experiments, in which they studied the reduced efficiency in labeling red cells when the chromate solution was premixed with plasma, show that the Cr^{51} was exposed not to plasma only but to a plasma-citrate-dextrose mixture. In the light of our experience it would appear that at least part of the effect they observed could be attributed to the citrate-dextrose mixture rather than to the plasma.

The impaired labeling in A.C.D. solution of blood samples with low hematocrit values is probably due to the fact that under these conditions the chance is reduced of a chromate ion entering a red cell and becoming bound there before being affected by the citrate-dextrose in the suspending solution. In saline suspensions of red cells, where no component of the suspending solution is capable of reacting with the chromate ion, a very large proportion of these ions will enter the red cells even when these are widely spaced in anemic blood.

Our experience indicates that the blood of anemic patients can be effectively labeled in three ways. The hematocrit may be adjusted to 40 or more by the removal of some of the plasma, or the cells may be washed and suspended in saline before the addition of the $Na_2Cr^{51}O_4$. Finally a larger dose of $Na_2Cr^{51}O_4$ may be added to the anemic blood, to allow for less efficient labeling, and the surplus radioactivity removed by washing the cells after incubation.

The accidental observation that admixture of $Na_2Cr^{51}O_4$ with A C D solution before the addition of red cells might lead to comparatively poor labeling is amply confirmed in the experiments designed to elucidate this finding. They show that a variety of influences may impair the ability of the chromate ion to enter red cells. The labeling power of $Na_2Cr^{51}O_4$ is adversely affected by decreasing the pH of the solution, by increasing the citrate concentration, and to a lesser extent by increases in concentration of dextrose. Whatever the nature of the reaction involved, it is accelerated by exposing the reaction mixture to light. Impairment occurs more rapidly with A C D (B T S) solution than with A C D (B P) solution. The concentrations of both citrate and dextrose are greater in A C D (B T S) solution than in A C D (B P) solution, and these differences are probably of significance. The methods of sterilizing these solutions also differ in detail. Autoclaving dextrose solution tends to cause caramelization with an increase in its reducing properties. The components of both A C D preparations are autoclaved but it would appear that the technique used by the B T S laboratory causes more caramelization. We have demonstrated that A C D (B P) solution is also capable of causing very rapid deterioration in the labeling power of $Na_2Cr^{51}O_4$ if visible caramelization is produced in it by heating to 100° C for 4 hours.

Anionic chromium in the form of $Na_2Cr^{51}O_4$ has a marked affinity for red cells and in the tracer amounts used in labeling techniques, under optimum conditions, it is almost entirely taken up by the red cells. Cationic chromium in the form of $Cr^{51}Cl_3$, on the other hand, enters the red cells only in negligible amounts if it does so at all. Thus reduction of the chromate to the chromic ion, as suggested by Cooper and Owen[2] offers a simple but probable explanation for the changes in $Na_2Cr^{51}O_4$ when it is mixed with A C D before the addition of red cells. Evidence that reduction of chromate to chromic ions by acid dextrose does occur was obtained both by tracer techniques and by spectrophotometric analysis of mixtures of acid dextrose with K_2CrO_4.

The formation of complexes between chromic ions and citrates precludes the use of the more direct methods of detecting the conversion of chromate to chromic ions, such as precipitation or trapping by an ion exchange resin to study the reaction between $Na_2Cr^{51}O_4$ and citrate solutions. However, the spectrophotometric analysis of mixtures of citrate and chromate solutions reveals, in time the development of an absorption band between 570 and 580 $m\mu$, this is of similar configuration to that found in the test solutions to which chromic ions have been added. The rate of development of this band appears to be influenced by the same factors as those which affect the labeling power of chromate. Thus it appears more rapidly in acid solutions exposed to the light and when the A C D solution has been partially caramelized by heating. There is a discrepancy in the case of disodium citrate which proved inactive against 1 per cent K_2CrO_4 in the spectrophotometric study, whereas in the tracer experiments it was almost as active as A C D solution in reducing the labeling power of chromate. This may well be explained by the buffering action of 1 per cent K_2CrO_4 solution which is probably sufficient to raise the pH of the disodium citrate solution to a value at which the reaction is very slow. The critical value for this purpose would appear to be about pH 6. At lower values a proportion of the chromate is converted to the more active dichromate and chromic acid.

The difficulty which we experienced in measuring the proportion of chromate ion reduced to the cationic state in citrate solutions raises the question of whether the biologic behavior of the chromic-citrate complex may differ from that of the free chromic ion which, as previously mentioned, becomes bound to the plasma proteins. Pilot experiments have been carried out to clarify this question. Serum was incubated with chromic chloride or with chromate, the power of which to label red cells had been destroyed by contact with A.C.D. solution. Electrophoretic analysis of the serum showed that the chromic chloride and the reduced chromate behave differently. Chromic chloride moves with the proteins, chiefly the albumin fraction, whereas the "reduced chromate" migrates very rapidly towards the anode, behaving as a free anion. Comparison of the behavior of chromic chloride and "reduced chromate" in vivo would be of interest.

CONCLUSIONS

Our studies have shed light on the change that occurs in the chromate ion when solutions of $Na_2Cr^{51}O_4$ and A.C.D. are mixed. There is good evidence that the chromate ion is reduced to the chromic state so that it can no longer enter the red cells, as suggested by Cooper and Owen.[2] Our investigations also suggest that a chromic-citrate complex forms, the biologic behavior of which is as yet unknown. Pilot electrophoretic studies show that chromic chloride and the chromic-citrate complex behave differently in vitro. The former moves with the proteins, whereas the latter migrates rapidly towards the anode.

Our experience suggests that it is not possible to keep stock mixtures of A.C.D. and $Na_2Cr^{51}O_4$ solutions for more than 4 hours, if efficient labeling is to be maintained. Even this period of premixing is possible only if the A.C.D. solution is not sterilized by autoclaving, and the mixture is not exposed to strong light.

SUMMARY

Prior admixture of $Na_2Cr^{51}O_4$ and A.C.D. solutions, which would enable a solution of A.C.D. labeled with Cr^{51} to be maintained for emergency use, impairs the ability of Cr^{51} to tag red cells.

The effect is more obvious with A.C.D. (B.T.S.) than with A.C.D. (B.P.) solution.

Labeling is adversely affected by the duration of prior mixing before the addition of red cells, by decreasing the pH of the solutions, by increasing the concentration of citrate and dextrose, by exposing the A.C.D and $Na_2Cr^{51}O_4$ mixture to light, and by autoclaving the dextrose solutions.

Inquiry into the nature of the change which occurs in the $Na_2Cr^{51}O_4$ has established that the chromate is reduced to the chromic ion The "reduced chromate" forms a firm complex with citrate and does not become bound to the plasma protein as does the free chromic ion Evidence is presented to show that it is not possible to keep a solution of A C D labeled with Cr^{51} and maintain efficient labeling for more than 4 hours

We wish to express our thanks to Professor L J Davis for his advice and criticisms We are grateful for the help of Dr Bell, Department of Chemistry, Glasgow University, and Dr Crawford, Royal College of Science and Technology, Glasgow, with whom we discussed the chemical aspects of the investigations

The radiochromium was supplied by the Regional Physics Centre, Glasgow, Scotland

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Thirtieth Annual Meeting

Chicago, Ill., Nov. 1 and 2, 1957

ABSTRACTS

1. COMPARISON OF THE INFLUENCE OF OPERATIVE TRAUMA AND NUTRITIONAL MANAGEMENT ON METABOLIC ALTERATIONS

WILLIAM E. ABBOTT, M.D., HARVEY KRIEGER, M.D. (BY INVITATION), AND STANLEY LEVEY, PH.D. (BY INVITATION), CLEVELAND, OHIO

In previous studies on patients of the same sex undergoing a gastrectomy it was noted that by providing adequate amounts of nitrogen, calories, electrolytes, and fluids, postoperative abnormalities in the metabolism of sodium, nitrogen, and potassium as well as loss of body weight could be minimized or negated. In order to eliminate the effect of variations resulting from different ages and states of nutrition and to assess the effect of operative stress versus diet, the following studies were carried out. The metabolic changes resulting from a 3-day control period of intravenous feeding were compared with alterations occurring following cholecystectomy when the patients were maintained on the same intravenous regimen as that given during the control period. Ten women were studied, all of whom underwent a cholecystectomy. Three were maintained solely on a 5 per cent dextrose solution in water during the control and 3-day postoperative period. Three additional subjects received a comparable regimen plus approximately 90 mEq. of both sodium and potassium. A third group of 4 women were given the latter regimen plus a 5 per cent protein hydrolysate. The daily excretion of urinary 17-hydroxycorticoids were within the normal range for both the control and oral repletion period. On the day of operation and the next two days there was an increase in excretion of the 17-hydroxycorticoids (50 to 300 per cent of the control value).

It may be concluded from the results obtained that while there was some variation from patient to patient in the metabolic alterations of the two periods the changes noted were largely due to the nutritional regimen employed and to a lesser degree to the stress of operation. Similar studies with another group of patients indicate that postoperative complications (hemorrhage, fever, etc.) or severe trauma in men evokes a greater metabolic response than that occurring in these women who underwent a cholecystectomy.

2. RELATIONSHIP BETWEEN A RANGE OF TISSUE TEMPERATURE AND LOCAL OXYGEN CONSUMPTION IN THE RESTING FOREARM

D. I. Abramson, M.D., A. Kahn (By Invitation), S. Tuck, Jr., B.S. (By Invitation), G. A. Turman, M.D. (By Invitation), H. Rejal, B.S. (By Invitation), and C. J. Fleischer, M.D. (By Invitation), Chicago, Ill.

With the introduction of hypothermia in surgery interest has been revived in the effects of temperature on tissue metabolism. Although work has been performed in the past on in vitro muscle slices and on the entire organism, the effects of changes in temperature on the oxygen consumption of specific tissues in vivo have not been reported. Such a study was attempted in the present investigation, involving 21 experiments on 13 normal subjects.

In each instance blood flow in the forearm was obtained using the venous occlusion plethysmograph, with the limb being exposed consecutively to water at 45°, 32°, and 17° C. Oxygen content of samples of blood, collected at each bath temperature from veins draining the tissues under study, was determined with the Van Slyke manometric method. Skin, subcutaneous tissue, and muscle temperatures were also recorded at each bath temperature, using thermocouples. *From the data on blood flow and oxygen determination, oxygen consumption* of the tissues of the forearm (except bone) was calculated.

At an average tissue temperature of 39.9° C. (produced by a bath temperature of 45° C.), the average oxygen consumption was 0.332 ml./min./100 ml. limb volume. At an average tissue temperature of 34.5° C. (produced by a bath temperature of 32° C.), the average oxygen consumption was 0.173 ml. At an average tissue temperature of 24.0° C. (produced by a bath temperature of 17° C.), the average oxygen consumption was 0.071 ml.

A statistical analysis of the variations of oxygen consumption as related to average tissue temperature revealed an overwhelmingly significant difference in the readings obtained at the three bath temperatures.

Therefore it was concluded that exposure of the tissues of the forearm to local heat produced a definite rise in oxygen consumption, while cold had the opposite effect. The Q_{10} of the oxygen consumption rate was found to have a mean value of 2.5.

3. EXOPHTHALMOMETRY IN GRAVES' DISEASE

A Twenty-year Study

Willard Bartlett, Jr., M.D., St. Louis, Mo.

Exophthalmometric measurements are reported on 75 patients treated for Graves' disease since 1936. The average period of serial observations is 11 years, the shortest is 2 years, and the longest is 20 years.

Analysis of Data on Proptosis.—(1) No characteristic change accompanied the uniform metabolic improvement during preoperative treatment. (2) Between preoperative and early (4 months) postoperative measurements, decrease occurred in 10 per cent. (3) Between either preoperative or early postoperative measurement and the final observation, decrease occurred in 16 per cent, increase in 74 per cent. (4) The range of final measurements in 59 instances reveals proptosis of 18 mm. or less (bilateral) in 15 per cent, of 20 mm. or less in 27 per cent. (5) In serial observations on 46 postoperative patients of constant therapeutic regimens, variation of proptosis of as little as 1 mm. was seen in only 2 patients, regardless of metabolic status.

Comment.—Individuals who have had Graves' disease show a wider variation of proptosis from time to time than do others. Cosmetic improvement reflects only narrowing of the palpebral fissure. Postoperative hypothyroidism is accompanied by exaggeration of the usual increase in proptosis, and this is prevented or reversed by adequate thyroid dosage, as stated by Ruedemann (1931) and the author (1932), even in early "malignant" exophthalmos.

These data do not support the views that there is a correlation between increase of proptosis and either the rapidity (Soley) or the amount (Dobyns) of reduction of metabolism.

Undoubtedly, many orbital decompressions have been performed without knowledge of an alternative. In this series, 5 patients with "malignant" exophthalmos have been seen and 4 have previously been cared for, none requiring orbital decompression since 1930.

Desiccated thyroid in small doses is being employed late in the preoperative regimen and is promptly increased after operation to maintenance dosage. Children are treated exactly as are adults. The author continues to reject the dictum that thyroidectomy should be avoided in the patient with severe exophthalmos and mild hyperthyroidism.

Menstrual histories of 50 patients in this series suggest strongly that menstruation persists to a higher age in women who have had Graves' disease than in others.

4. PULMONARY FUNCTION IN OBESE PERSONS

George N. Bedell, M.D. (By Invitation), William R. Wilson, M.D. (By Invitation), and Paul M. Seebohm, M.D., Iowa City, Iowa

Numerous medical reports have appeared calling attention to a syndrome of obesity, arterial hypoxemia, alveolar hypoventilation, and polycythemia. This study was undertaken to demonstrate (1) the frequency of this syndrome in extremely obese persons, and (2) the mechanism of hypoxemia. Pulmonary function studies have been done in 22 obese persons and 14 of these have been restudied following weight loss.

The patients are divided into 2 groups depending on arterial oxygen saturation at rest. Twelve patients (average weight 300 pounds) with normal saturation were put in Group I. These patients had normal lung volumes; mean minute volume of ventilation of 9.9 L., mean alveolar ventilation of 6.3 L., mean physiologic dead space of 215 ml., slightly uneven distribution of inspired air, mean maximal breathing capacity (MBC) of 86 per cent of predicted normal; mean maximal expiratory flow rate (MEFR) of 209 L./min., mean maximal inspiratory flow rate (MIFR) of 154 L./min., and normal diffusion. Five patients restudied after a mean loss of 54 pounds had an increase in mean expiratory reserve volume (ERV) from 64 per cent to 106 per cent, increase in mean MBC from 76 per cent to 91 per cent, increase in mean MEFR from 180 L./min. to 199 L./min., and MIFR from 170 L./min. to 194 L./min.

Ten obese patients (average weight 315 pounds) with arterial hypoxemia compose Group II. Nine of the 10 had clinical or laboratory evidence of lung disease One patient had arterial hypoxemia (90 2 per cent) and no evidence of lung disease Following weight reduction some of these patients had improved pulmonary function

We conclude that in most obese persons hypoxemia is secondary to lung disease and not caused by obesity alone Obesity in persons with normal lungs causes a decrease in ERV, MBC, MEFR, and MIFR, but is partly reversible by weight reduction

5 OBSERVATIONS ON POTASSIUM REQUIREMENTS IN MAN

William B Bean, M D, and Robert Hodges, M D (By Invitation),
Iowa City, Iowa

There are no long term studies of potassium requirements in man Often it is stated that any natural diet has enough potassium In recent experiments on pantothenic acid metabolism, 6 normal young men were maintained on a purified, partly synthetic diet containing 800 mg of potassium a day expressed as potassium ions Three of the 6 received an additional gram of potassium chloride daily One patient with added potassium and one without were given only the standard diet and a standard vitamin and mineral supplement Another pair had the same regimen but pantothenic acid was removed, and the remaining pair had large doses of a pantothenic acid antagonist omegamethyl pantothenic acid The experimental period of 10 weeks followed a 4 week control period There was a similar recovery period The urinary excretion of potassium fell rapidly in all subjects on the experimental diet and became steady at a lower level after two weeks, ranging from 10 to 20 mEq a day On *many occasions the serum potassium of those without supplements fell below* our lower limit of normal but symptoms of hypokalemia did not appear Serum potassium remained just at or below the lower limits of normal In each subject with supplemental potassium, the urinary levels were a little higher and the blood levels within the normal range Though there was a slight rise in blood CO_2, alkalosis did not develop

These observations indicate that an intake of 800 mg of potassium ions is about the minimal daily requirement and that an additional gram a day is adequate, and perhaps ample

6 METABOLIC EFFECTS OF HUMAN AND MONKEY GROWTH HORMONE IN MAN

Delbert M Bergenstal, M D, Herbert A Lubs, M D (By Invitation), Lois F Hallman (By Invitation), James Patten, M D (By Invitation), Herbert J Levine, M D (By Invitation), and C H Li, M D (By Invitation),
Bethesda, Md

Growth hormone prepared from human and monkey pituitary glands by C H Li has been shown to have differences in chemical structure and physical properties from that prepared from beef pituitary glands These growth hormone preparations were administered to 3 patients under metabolic balance conditions At dose levels from 2 5 to 10 mg a day, marked metabolic effects were observed including positive nitrogen, phosphorus balances, and altered electrolyte balances By use of intravenous glucose insulin tolerance tests a definite insulin resistance was observed One patient with persistent lactation demonstrated a marked increase in lactation during growth hormone administration. A comparative study of monkey and human growth hormone was made in a hypopituitary patient which showed greater activity of the human preparation than the monkey preparation. The details of the metabolic balance results will be presented. There was no evidence of adrenocortical stimulation and only slight thyroid stimulation by these hormones. Measurement of aminopolysaccharides in the urine in a hypophysectomized patient with chondrosarcoma resulted in an increase of this material in the urine during growth hormone administration and this probably reflects a stimulation of the formation of mucopolysaccharides by the tumor since cartilage tissue is extremely sensitive to growth hormone. The relationships of the differences in structure between beef and human growth hormone in reference to its metabolic activity will be discussed.

7. AN ATHYROTIC MOTHER WITH TWO CRETINOUS CHILDREN WITH THYROID GLANDS

William H. Beierwaltes, M.D., E. A. Carr, Jr., M.D. (By Invitation), V. N. Dodson, M.D. (By Invitation), G. H. Lowrey, M.D. (By Invitation), A. H. Wheeler, Ph.D. (By Invitation), and N. Spafford, A.B. (By Invitation), Ann Arbor, Mich.

Most cretins are athyrotic and their mothers are euthyroid. The cause of thyroid defects resulting in cretinism is unknown. To our knowledge, no one has reported sibling cretins with thyroid glands born to an athyrotic mother, nor has anyone suggested that cretins might acquire thyroid damage from maternal or fetal thyroid autoantibodies.

A clinically hypothyroid 26-year-old mother and her 3-year-old and 2-year-old cretins were studied. The mother's 1-, 2-, 5-, and 24-hour thyroid I^{131} uptakes, all under 3 per cent, the scintiscan of her neck, and her B.M.R. of −32 per cent were compatible with total absence of thyroid gland. She regularly had taken 90 to 192 mg. of thyroid per day throughout both pregnancies. The cretins, at University Hospital before 6 months of age, had demonstrated classic physical and roentgenographic stigmata of cretinism, hypothyroid I^{131} uptakes, and a classic response to the subsequent administration of desiccated thyroid. Off thyroid for 6 weeks, the children both attained normal I^{131} uptakes during the 1- to 5-hour intervals but subnormal 24-hour uptakes. The 3-year-old demonstrated an elevated PBI^{131} at 24 hours, and the 2-year-old a high normal PBI and low normal serum cholesterol. Paper chromatography of serum after I^{131} demonstrated significant quantities of thyroxine in both cretins, triiodothyronine in the younger and no diiodotyrosine in either. Precipitin and complement fixation tests of the sera of this mother and these children and of 11 other mothers of 11 other proved cretins 6 weeks to 13 years of age have not demonstrated human thyroid antoantibodies to date. We have, however, demonstrated high human thyroid autoantibody titers in human Hashimoto's struma, and we have demonstrably "immunized" rabbits to human thyroid antigen.

These findings suggest that: (1) cretinism in the human is not a result of a low level of maternal thyroid hormone; (2) a child may be born with "normal" but too little thyroid gland to maintain adequate body growth; and (3) there is no proof, to date, that thyroid autoantibodies of mother or child are the cause of thyroid defects of cretinism.

8 OBSERVATIONS ON INTRINSIC FACTOR AND THE INTESTINAL ABSORPTION OF VITAMIN B_{12}

WILLIAM R BEST, M D, JOHN H FRENSTER, M D (BY INVITATION), AND
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$B_{12}Co^{60}$ can be absorbed when placed by tube with intrinsic factor concentrate (IFC) in the stomach, duodenum (best absorption), jejunum, or ileum (Fed Proc **16** 353, 1957) A patient with pernicious anemia had 8 9 per cent 24 hour urinary excretion when 1 0 µg $B_{12}Co^{60}$ was given with 1 0 U S P unit IFC by tube into the proximal ileum followed by 1 0 mg B_{12} parenterally Peak excretion of 1 05 per cent/hr occurred at 8 to 10 hours, the same time as after oral administration *Thus, the main delay in transport of B_{12} from stomach to blood stream is not due to intestinal transit time, but must be related to mucosal cell processes* When $B_{12}Co^{60}$ was similarly given into the mid jejunum without IFC, only 0 6 per cent was excreted, confirming that protection of B_{12} from inactivating forces high in the intestine is not the mode of IFC action

Another PA patient received 0 5 unit IFC with 1 0 µg $B_{12}Co^{60}$ and 100 ml thin barium through a tube just proximal to a balloon obstructing the upper jejunum One milligram of B_{12} was given parenterally at 0, 12, and 24 hours The balloon was released at 12 hours (material was retained in duodenum and proximal jejunum by x ray) Twenty one hundredths per cent $B_{12}Co^{60}$ was excreted during the first, 0 15 per cent during the second, and 0 27 per cent during the third 12 hour periods The same mixture orally with the same injections gave 3 9 per cent during the first, 8 2 per cent during the second and 2 6 per cent during the third 12 hour periods Barium was in the terminal ileum and colon by 12 hours Thus *vitamin B_{12} cannot be absorbed by the upper small bowel, and, as a matter of fact, IFC activity is destroyed by too long a sojurn there*

Cellular enzymes undoubtedly act (with IFC help) in the transport of B_{12} from gut lumen into circulation Carbonic anhydrase might conceivably be involved Two hundred fifty milligrams acetazolamide (Diamox) was given every 8 hours for 3 days to 2 patients with pernicious anemia Schilling tests using 1 unit IFC in one patient and 0 5 unit in the other gave 13 8 per cent and 10 5 per cent excretions, respectively before, and 16 7 per cent and 10 5 per cent on the third day of acetazolamide administration Thus, *carbonic anhydrase is not implicated*

9 ELECTROPHORETIC STUDIES OF THE SERUM PROTEINS IN ACTIVE ULCERATIVE COLITIS

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The serum proteins were studied serially by filter paper electrophoresis in 23 patients with active ulcerative colitis For comparison, normal individuals and patients with rheumatoid arthritis, systemic lupus erythematosus, cirrhosis of the liver, multiple myeloma, nontropical sprue, regional ileitis, and healed ulcerative colitis were studied similarly

The protein partition in active ulcerative colitis followed two characteristic patterns, most commonly, hypoalbuminemia and elevations of alpha 2 and gamma globulin (15 patients); less frequently, hypoalbuminemia and elevation of alpha-2 globulin only (8 patients). The first pattern has been described in collagen diseases and resembles that observed in patients with rheumatoid arthritis, scleroderma, and systemic lupus.

The effect of standard medical treatment, often supplemented with corticotropin or adrenal steroids, upon the electrophoretic patterns also was studied serially. The clinical response in those patients with an elevated gamma globulin was prompt; the electrophoretic response was delayed but eventually the pattern became normal. In two patients "T" globulins were seen but these disappeared with steroid therapy; the clinical response to steroids in these cases was dramatic. In the second group, i.e., hypoalbuminemia with elevated alpha-2 only, steroids were considerably less effective; large amounts were required to achieve any clinical response and complications of such therapy were more frequent.

Steroids do not influence antibody formation but they interfere with antigen-antibody reactions of the hypersensitivity type. The response to steroids in ulcerative colitis and the accompanying return of the abnormal protein patterns toward normal are in accord with the concept of a hypersensitivity mechanism in active ulcerative colitis. Electrophoretic patterns of the serum proteins apparently help to select patients who will respond to steroids and thus provide a more rational basis for their use. The "T" globulins found in two patients suggest that an immunologic mechanism also may be involved in the pathogenesis of the disease.

10. THE GENETIC MECHANISM OF IDIOPATHIC HYPERLIPEMIA

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(Introduced by Ralph Dolkart, M.D.)

The propositus, a 9-year-old girl of Puerto Rican origin, was first seen because of multiple xanthomata. Subsequently, she developed precordial pain, and died suddenly from cardiac complications. On post-mortem examination, there was atherosclerosis involving the endocardium and the medium- and large-sized arteries, including the blood vessels of the brain. Death was due to occlusion of the coronary vessels and infarction of the myocardium.

Serum samples were obtained from all members of the immediate family on two separate occasions. These were analyzed for total lipids, phospholipids, cholesterol, and cholesterol esters. In addition, the serum proteins and lipoproteins were separated by paper electrophoresis. The following changes were found: (1) In addition to the propositus, two asymptomatic siblings were found to have all of the characteristic changes of idiopathic hyperlipemia. (2) Both parents and two additional siblings showed elevations of total and esterified cholesterols. (3) The three affected children showed a virtual absence of the alpha liproproteins, suggesting that the basic defect in the disease process lies in an inability to convert beta liproproteins to alpha lipoproteins. This could possibly occur because of a genetically determined enzyme deficiency. (4) A bright blue hue is present over the beta lipoprotein in all three affected children. (5) An abnormal fluorescent, narrow band is seen over the beta globulin area in two affected children and under the gamma globulin area in the third child.

It is generally agreed that a recessive mode of inheritance is suggested if the following criteria are present: (1) The presence of the condition in siblings, but not among ancestral or collateral relations. This has been shown in the present pedigree (2) If the condition is present in monozygotic, but not in dizygotic twins This could not be tested in the present family (3) If there is a history of consanguinity in the family The parents of this family are first cousins (4) If the heterozygous carriers of the abnormal gene are detectable by means of laboratory tests This has been shown in this family

This indicates that idiopathic hyperlipemia should be classified among the "inborn errors of metabolism" and the condition appears to be transmitted by a recessive gene

11 ACID MUCOPOLYSACCHARIDES IN PLEURAL, PERICARDIAL, AND ASCITIC FLUIDS

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and William F Simpson, M S (By Invitation), Detroit, Mich

Polyserositis is a frequent occurrence in systemic connective tissue diseases Since connective tissue is characterized by a high content of acid mucopolysaccharides, it was decided to determine whether these substances are present in effusions in serous cavities Pleural, pericardial, and ascitic fluid from 14 patients was studied, and found to contain acid mucopolysaccharide The concentration ranged from 44 to 1,470 μg per cent, measured as uronic acid by the carbazole method The highest levels were observed in a pleural and a pericardial fluid from two patients with systemic lupus erythematosus, and ascitic fluid from a patient with carcinomatosis In general, the lowest levels were found in fluid from patients with congestive heart failure and cirrhosis of the liver Following incubation with testicular hyaluronidase, acid mucopolysaccharide could no longer be detected by the methods used

Chromatographic studies of the acid mucopolysaccharide isolated from several fluids, using a propanol phosphate buffer system, revealed the presence of two components staining metachromatically One spot had the mobility of chondroitin sulfate and heparin, which are not separated by this solvent The second spot remained at the origin, as did samples of hyaluronic acid All the fluid examined in this fashion showed the same two components Plasma samples obtained from these patients as well as many other individuals have revealed the presence of components with the chromatographic mobility of chondroitin sulfate and heparin, but none resembling hyaluronic acid In addition, the hexosamine isolated from acid mucopolysaccharide of ascitic fluid was found to consist of 58 per cent glucosamine and 42 per cent galactosamine, whereas that from plasma contained mainly galactosamine Hyaluronic acid has been reported to be present in relatively high concentration in fluid from a patient with mesothelioma of the pleura

It is concluded from this data that at least one acid mucopolysaccharide present in pleural, pericardia, and ascitic fluid is derived from the lining membrane of these serous cavities Chromatographic studies and the relatively high glucosamine content suggest that it is hyaluronic acid

12 THE USE OF A SALINE CONDUCTIVITY METHOD FOR THE DETERMINATION OF CARDIAC SHUNTS BY THE INDICATOR DILUTION TECHNIQUE

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The indicator dilution technique is an accepted method for the determination of the site and type of cardiovascular shunting The most commonly employed indicator has been Evans blue with the dye curves being recorded photometrically.

A new method has been developed for this technique, utilizing the principle of a saline induced increase in blood electrical conductivity. The indicator, 5 per cent saline, is injected into different sites in the right heart during cardiac catheterization and the dilution curves are obtained from systemic arterial blood drawn through the conductivity cell, which is a hollow plastic cylinder with two imbedded platinum electrodes. The remaining instrumentation consists of a simple modification of any commonly used carrier amplifier plus a standard recorder.

This method has been used successfully in 21 patients with congenital heart disease, 12 of whom had shunts. Its validity has been established by: (1) simultaneous comparison with dye curves, (2) catheterization data, (3) cardiac surgery, and (4) autopsy. Representative curves from 4 patients will be shown as follows: (1) a pulmonic stenosis without shunting, (2) an Eisenmenger complex with a right-to-left shunt where simultaneous dye and conductivity curves showed identical contours and time bases, (3) a ventricular septal defect where four curves, one from the pulmonary artery and three from the right ventricle showed bidirectional but predominately left-to-right shunting, and (4) a tetralogy of Fallot with a curve from the right ventricle showing a right-to-left shunt.

The method retains the desirable feature of the Evans blue technique in that the curve is immediately available. It possesses, in addition, the following advantages: (1) determinations can be made as frequently as desired because there is no appreciable accumulation of indicator resulting in increased background in the blood; (2) saline does not discolor the patient; (3) saline does not interfere with cuvette oximetry as Evans blue does; therefore, curves can be obtained at any time during cardiac catheterization; and (4) most of the necessary instrumentation is accomplished by the simple and inexpensive modification of commonly used amplifier-recording systems.

13. THE FUNCTIONAL CAPACITY OF PERSISTING NEPHRONS IN EXPERIMENTALLY INDUCED CHRONIC BRIGHT'S DISEASE

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The contention is widely held that the kidney with chronic Bright's disease is no longer a "true" kidney in that its constituent nephrons are disorganized functionally as well as morphologically. Using a method previously described, permanent unilateral renal disease has been induced in dogs. All damaged nephrons are thereby confined to one kidney. Comparison of the simultaneous functions of both kidneys under a variety of experimental conditions has allowed examination of the integrity of the persisting nephrons in the altered kidney.

Within two to four months after the experimental procedure (perfusion of the anoxic kidney with 2,6-dimethylaminopurine-3-amino-d-ribose, diverting renal venous blood to prevent recirculation), the involved kidneys become contracted, often to one-fourth their original size. Clinically, the animals remain healthy and develop neither hypertension nor progressive anemia. Histologically, the diseased kidneys show sclerosis of glomeruli, dilation, and atrophy of tubules and fibrosis. Intact kidneys have remained normal.

Functionally (GFR, ERPF, Tm_{PAH}, and $Tm_{glucose}$), the diseased kidneys show marked and persistent impairment, indistinguishable from contracted kidneys of human Bright's disease. After an initial increase, the normal kidneys remain at approximately control levels of function.

Consistency of clearance values (after stabilization) for prolonged periods plus comparability of values for C_{Cr}/C_{PAH}, C_{Cr}/Tm_{PAH} and $C_{Cr}/Tm_{glucose}$ between the two kidneys, support the validity of clearance techniques in studying the diseased kidneys. These same data attest to the integrity of the persisting nephrons in the contracted kidney. Thus the volume of glomerular filtrate per unit of effective renal plasma flow and glomerulotubular balance (C_{Cr}/Tm_{PAH} and $C_{Cr}/Tm_{glucose}$) have remained equal bilaterally although GT balance changed in both kidneys after induction of unilateral lesions such that GFR per nephron increased. This suggests that functioning nephrons in both normal and diseased kidneys adapt to a decrease in total nephron population by increasing GFR. The hypothesis that this is the major adaptive change occurring in the contracted kidney is currently being evaluated.

It is concluded that nephrons in the contracted kidney are reduced in number but possess normal functional characteristics.

14. THE COLLATERAL CIRCULATION IN CORONARY HEART DISEASE: "BENIGN" or "MALIGNANT"?

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(Introduced by Harry Goldblatt, M.D.)

In view of the absence of any obligate correlation between the location and extent of the occlusive process in the coronary arteries and the clinical-pathologic consequences thereof, our comprehension of fundamental pathophysiologic relationships in this disease has particularly suffered from a natural (and profound) reluctance (for fear of untoward reactions) to perform such vital studies in patients with clinical coronary heart disease. Consequently, a priori reasoning and empirical concepts abound.

This presentation is based upon a unique opportunity for *direct* hemodynamic and metabolic observations on the heart in more than 300 patients with *clinical* evidence of coronary heart disease operated upon by Dr. Claude Beck. A large control group was provided by over 500 patients undergoing thoracic surgery for other than coronary heart disease. In these studies, direct insertion of a catheter into the coronary sinus (or a suitable coronary vein) permitted serial determinations of the myocardial extraction coefficient ($\frac{A\text{-}V}{A}$) for O_2 and various other substrates. From these studies has emerged a more or less unexpected observation: Generally, there was no definite *increase* in oxygen extraction, as would be anticipated a priori in patients with coronary heart disease; on the contrary, particularly in patients with extensive myocardial scarring, there was a significant *decrease* (*increased* coronary venous O_2 content). This apparently paradoxical situation is further confirmed by experimental, clinical, and anatomic observations, and should stimulate a critical reappraisal of many empirical and old "established" concepts in coronary heart disease. With adequate collaterals to provide uniform distribution via a final common pathway for the ubiquitous red blood cell, the integrity of the myocardium may be maintained almost indefinitely despite multiple complete coronary artery occlusions. However achieved, intercoronary anastomoses *in advance* of the occlusive process are truly beneficial; the extensive collaterals resulting from coronary occlusion and myocardial infarction are not necessarily so, and may actually be deleterious in that they encourage perfusion of a low-resistance scar. This preferential flow then deprives adjacent viable myocardium of critically needed blood.

15. LEVELS OF ANTIBODY AGAINST DENVER A'-1957 AND ASIAN-1957 INFLUENZA VIRUSES IN HUMAN AND ANIMAL SERA IN AN AREA NOT YET INVOLVED IN AN EPIDEMIC OUTBREAK

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Observation of the incidence of influenza in the area of St. Louis, Mo., in the past 14 years by clinical and laboratory studies permits an evaluation of changes in antibody levels occurring in the population in recent months. Studies of paired sera, culture of throat washings, examination of sera of infants, and of serum pools collected according to date of birth give evidence concerning the prevalence of recently isolated strains of influenza virus in this area.

There is evidence that an A' strain isolated in Colorado by Dr. Meikeljohn in January, 1957, in all probability reached the St. Louis area within a month after its isolation but without causing a clinically recognized outbreak. Only a few cases showed antibody rises against this virus with accompanying clinical symptoms of acute influenza. Evidence for the presence of the virus in the area is found by examination of infant sera collected consecutively by months, during the winter and spring. This virus shows some cross reactions with A' strains isolated in St. Louis in 1953. Individuals born between 1895 and 1905 show the highest levels of "natural" antibodies against the Denver virus. Animal sera collected in this area showed antibodies against this strain as soon as and perhaps even earlier than human sera collected simultaneously.

Antihemagglutinins against the Asian strain which had been passed only in eggs were found in but a single 80-year-old individual in the St. Louis area up to Sept. 1, 1957. He had no evidence of clinical influenza. The strain passed through mice has been much more reactive. Highest antibody titers have been found in those over 60 years of age. Examination of throat washings and paired sera from "influenza-like" illness in this area up to Sept. 1, 1957, have shown no definite evidence that the infection has spread to this vicinity. Animal sera collected up to this date show no antibodies against the Asian strain of virus.

16. SUGGESTED REVISION OF NOMENCLATURE FOR INTRAVENTRICULAR CONDUCTION "DEFECTS"

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Practically all types of so-called defects of intraventricular conduction have been demonstrated by the electrocardiograms of healthy young subjects. The conventional pathologic connotation of the term "bundle branch block" makes a more objective and purely descriptive nomenclature desirable to aid in breaking with the concept that electrocardiographic abnormalities of themselves are diseases. Such a change would serve as a step toward reducing the incidence of electrocardiographic-induced iatrogenic heart disease.

A comparative study of 200 healthy young subjects and 100 older cardiac patients serves as the basis for suggesting a revision of electrocardiographic nomenclature. Based on the form of the QRS complex, "normal intraventricular conduction" (classic concept) occurred in 2 per cent of healthy subjects and 50 per cent of cardiac patients, "right bundle branch block" in 95 per cent of healthy subjects and 22 per cent of cardiac patients, "left bundle branch block" in 13 per cent of healthy subjects and 22 per cent of cardiac patients and "bilateral bundle branch block" in 10 per cent of healthy subjects and 3 per cent of cardiac patients. The QRS interval is of little value in assessing the presence or degree of intraventricular conduction "defects," and was found to be shorter, on the average, in cardiac patients than in healthy subjects.

A new nomenclature, based on the form of the QRS complex in right sided unipolar leads and modified by the value of the QRS interval, is suggested. The terminology of the American Heart Association is used. Thus "RS type" for the classic type of "normal intraventricular conduction," "RSR type" for "right bundle branch block," "QS type" for "left bundle branch block," and "QRSR type" for "bilateral bundle branch block."

17 REFLECTIONS OF THE AGING PROCESS IN MAN IN HIS SPATIAL VECTORCARDIOGRAM

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With aging, gradual and subtle progressive changes develop in the tissues, including the cardiovascular system. It was thought that such progressive changes with aging may be reflected in the spatial vectorcardiogram (sVCG), which records electrical events associated with the heartbeat in greater detail than the conventionally recorded ECG.

The sVCG was recorded in 224 normal subjects varying in age from 20 to 72 years. A close similarity was noted for all age groups in magnitude and spatial orientation of the maximal mean instantaneous vectors of the QRS and T sÊ loops with horizontal orientation being greater in the older people. The QRS sÊ loop progressively and subtly developed "distortions" which rose from an incidence of 22 per cent in the subjects of the third decade of life to 88 per cent in those of the seventh decade of life.

The significance and mechanisms of these changes remain unknown, but it would appear that they reflect in large part progressive alterations in the order of depolarization with aging, a newly noted expression of senescence. A more sensitive galvanometer and higher paper speed would record electrocardiographically some of the alterations. Such myocardial electrical alterations with age may prove to be clinically useful now that some may be objectively recorded.

18 A SIMPLE, RAPID, AND ACCURATE T-1824 EXTRACTION METHOD SUITABLE FOR ROUTINE MEASUREMENTS OF BLOOD VOLUME

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Methods for measuring blood volume have required special equipment or special care, and usually both, and have not been suitable for use in the average hospital laboratory. Of the methods available the dye methods come nearest to being satisfactory, but direct measurement of the optical density of dyed plasma requires skill and is rendered inaccurate by hemolysis and varying opalescence of the samples, and none of the described extraction methods are without their difficulties. T. H. Allen's method (1951, 1953), in which T-1824 is displaced from albumin by detergent, adsorbed to cellulose, and eluted into acetone-water is the best of the latter, but requires 3 hours for the extraction of the dye, too long a period for its use in the management of the emergency patient. We have modified Allen's method as follows. One to four milliliters of dyed plasma is treated with detergent in sodium phosphate solution. The mixture is passed through a wood pulp column (Solka Floc S.W 40A) and the adsorbed dye is freed from protein and pigments by a wash of the phosphate solution. The dye is then eluted with acetone-water, buffered, and the optical density is read in a Coleman Junior or other simple spectrophotometer. A standard prepared in the patient's blank plasma is put through the same process. Ninety-seven ± 2 per cent of the dye is extracted in 20 minutes at room temperature, the optical density of the plasma blanks is zero, and hemolysis, bilirubinemia, and opalescence do not interfere. Measurements made by this method show satisfactory agreement with those made by the direct method. The method seems applicable to all patients. Since results can be obtained 60 to 70 minutes after dye injection, it should prove particularly useful for the management of patients suffering from hemorrhage.

19. CORRECTION OF DEFECTS IN CLOTTING ACCELERATOR FACTORS BY ADMINISTRATION OF A NEW SULFHYDRYL-SUBSTITUTED METHYLNAPHTHOQUINONE, VITAMIN K-S(II)

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Two patients, critically ill with severe hemorrhagic disorders caused by a deficiency of accelerator factor(s) of blood coagulation, will be presented. One has been studied intensively for over six years. The deficiencies were corrected and the bleeding was controlled promptly and completely by the oral and/or intravenous administration of S-(2-methyl-1, 4-naphthoquinonyl-3)-β-mercaptopropionic acid, designated vitamin K-S(II). Banked and fresh blood in both patients and massive doses of vitamin K and K_1 in one patient were ineffective.

The design and preparation of vitamin K-S(II) was based upon experimental studies which showed that the accelerator activity in blood coagulation is influenced strikingly by the concomitant administration of DL-methionine and vitamin K. Because of the synergistic effect of methionine and vitamin K, vitamin K-S(II) was designed to incorporate both sulfur and propionic acid, a carbon chain product of methionine degradation, into the vitamin K molecule in the 3-position. Experimental and clinical studies in normal dogs and rats, cholecystnephrostomized dogs on normal, protein, or methionine-free diets, partially hepatectomized rats, and in patients have shown the vitamin K-S(II) to be fully as effective as the methionine plus vitamin K.

20. SHORTENED SURVIVAL OF INCOMPATIBLE RED CELLS IN PATIENTS WITH "AGAMMAGLOBULINEMIA": A SENSITIVE TEST FOR THE PRESENCE OF TRACES OF ISOANTIBODY

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Patients with agammaglobulinemia have been characterized as "universal recipients" because of the absence of anti-A and anti-B isoagglutinins from their sera. The present report describes the survival of ABO-*incompatible* red cells in the circulations of 4 adult patients whose sera showed an absence of gamma globulin on paper electrophoresis.

Although routine tests for anti-A and anti-B isoagglutinins were negative, more sensitive tests revealed very weak isoagglutinins in 3 of the 4 patients. Ten milliliters of Cr^{51}-tagged incompatible red cells were injected intravenously over a 2-minute interval. No untoward symptoms or signs were observed. Red cell survivals were shortened in all 4 patients, with Cr^{51} half-times from less than 10 minutes to as long as 9 days (Normal: 30 ± 3 days).

Although the in vitro tests for antibody activity revealed no qualitative differences among the sera from the 3 patients with detectable isoagglutinins, surface scintillation counting over the hepatic and splenic areas suggested two different mechanisms for red cell removal. One mechanism (in two patients) entailed nearly equal participation by both liver and spleen; the other (in one patient) was almost exclusively a function of the spleen.

The sensitivity of the above-described in vivo studies for the detection of minute amounts of circulating antibody was illustrated by a second study performed on one of the patients. Ten milliliters of Cr^{51}-tagged incompatible blood was injected and the rapid disappearance of the red cells (less than 10 per cent survival after 10 minutes) confirmed as described previously. One hour later, a pint of Cr^{51}-tagged blood from the same donor was transfused; 90 per cent of the incompatible cells were present in the recipient's circulation at the conclusion of the transfusion. There was no measurable destruction of the surviving red cells during the succeeding 24 hours, and their survival was followed during the subsequent 6 weeks by both the Ashby and the Cr^{51} methods, along with surface scintillation counting over the liver and spleen. The results provide an interesting comparison of the destruction of red cells under conditions of antibody excess compared to antibody insufficiency.

21. PEREGRINATING PROBLEM PATIENTS: "MUNCHAUSEN'S SYNDROME"

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(INTRODUCED BY ROBERT C. HARDIN, M.D.)

Not before reported in this country is an instance of "Munchausen's syndrome," so named by Richard Asher of England. The term was suggested by similarities between the bizarre symptoms and behavior exhibited by certain patients and the tales of wild adventure and personal achievement told by the fabulous Baron Munchausen. Patients with Munchausen's syndrome travel widely among hospitals feigning symptoms and producing factitious lesions which require emergency care or extensive diagnostic investigations. One such patient was admitted to the University of Iowa Hospitals, Iowa City, Iowa, August 18, 1954, because of hemoptysis and thrombophlebitis.

In the following days there unfolded a series of remarkable events starting with plausible symptoms and progressing through inappropriate behavior to uncontrollable violence and fantastic self-inflected wounds. Disposition was finally made to a state mental hospital from which he subsequently fled.

By correspondence, it was learned that this was a repetitive pattern which the patient had been following for several years, and which continued after leaving Iowa. In the months following he was admitted to at least thirteen hospitals from California to New York with similar occurrences in all. The admitting diagnosis has always been thrombophlebitis and pulmonary embolus, while his discharge diagnoses have included a variety of psychiatric and other disorders. His method of producing hemoptysis has not been determined despite extensive examinations. In the past he has had many surgical procedures including inferior vena cava ligation, which partly accounts for the continuing signs of thrombophlebitis. He always concocts a farfetched story to explain the abdominal scar, and refuses to cooperate in treatment.

This patient is representative of one of the subgroups of the syndrome which has been called the bleeding type or "hemorrhagica histrionica." He also has features of the abdominal type or "laparotomophilia migrans."

These patients are so nearly alike in their behavior that it seems useful to classify them as a group, using the descriptive term "Munchausen syndrome" so long as the exact psychiatric diagnosis remains unknown. It is thought that many such patients exist, although hitherto not recognized in this country.

22. PULMONARY CAPILLARY FLOW-PRESSURE RELATIONSHIPS

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(Introduced by Erwin Levin, M.D.)

Studies have been done employing a triple lumen cardiac catheter with an inflatable cuff allowing pressure measurements and blood aspiration proximal and distal to the point of occlusion. "Wedge" pressures and samples were also obtained by standard techniques. Since wedged and distal pressures were identical both in level and configuration, the latter were used when standard "P. cap." was unobtainable due to pulmonary hypertension.

Pressures distal to occlusion were normal in patients with no disease or those with localized pulmonary disease and elevated with mitral valve or left ventricular involvement. Diffuse pulmonary disease produced marked variations in measured distal or "P. cap." pressures coincident with the respiratory cycle reaching high levels in the expiratory phase. Aspiration of wedged or distal blood samples was easily accomplished in normal patients or those with heart disease but difficult or impossible in many with chronic pulmonary disease. When obtainable, this blood was more highly oxygenated than arterial blood having been exposed longer to alveolar air and, therefore, more closely equilibrated. Increase in pulmonary artery pressure can be readily observed with exercise, however, simultaneous measurement of distal pressure showed a proportional increase without any presumed rise in left atrial pressure.

It is concluded that when blood flow is arrested in portions of the pulmonary vascular bed, the static "lacunar" pressure beyond the occlusion is closely related to left atrial pressure particularly when elevated. Under circumstances of increased intrapulmonic pressure, "lacunar" pressures reflect this change rather than left atrial. It is quite possible that increased intrapulmonic pressure may collapse pulmonary capillaries if it exceeds left atrial pressure causing what might be termed a "retrograde block."

Evidence is presented that ordinary "wedge" pressures, particularly in patients with primary pulmonary disease, are not reliable indicators of true pulmonary capillary pressure and may lead to completely invalid conclusions regarding left atrial hemodynamics. These studies support the contention that regulation of pulmonary blood flow is extravascular unlike the intrinsic arteriolar control of the periphery.

23 CLINICAL OBSERVATIONS ON THE USE OF NOVOBIOCIN IN PENICILLIN RESISTANT STAPHYLOCOCCAL SEPTICEMIA

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E L Quinn M D Detroit, Mich

(Introduced by R W Smith, M D)

The seriousness of staphylococcal septicemia is indicated by a continuing mortality rate of 50 to 80 per cent despite modern antimicrobial therapy Thus, new antimicrobial agents demonstrating antistaphylococcal activity are worthy of careful consideration and study

Novobiocin, an antibiotic agent derived from *Streptomyces niveus* and demonstrating both in vitro and in vivo antistaphylococcal activity, became available for investigational study approximately two years ago Subsequent investigations in our own and other laboratories indicated that this agent might prove useful in the treatment of staphylococcal sepsis due to penicillin resistant organisms

During the past 20 months, 7 patients with staphylococcal sepsis have been treated with novobiocin The following significant features were common to all cases and preceded the onset of staphylococcal sepsis in all cases an underlying primary disease process, hospitalization surgical intervention, and previous exposure to various antimicrobial agents In each instance the isolated strains of staphylococci were pigmented, hemolytic and coagulase positive

Five of the 7 patients responded satisfactorily to treatment with novobiocin Of the remaining 2 cases, 1 developed severe thrombocytopenia purpura, a previously unreported toxic reaction, on the ninth day of therapy after receiving 18 Gm of novobiocin In the other patient emergence of a novobiocin resistant strain of staphylococcus occurred after 7 days of therapy

24 REVERSAL OF THE LESIONS OF THE GENERALIZED SHWARTZMAN PHENOMENON BY TREATMENT OF RABBITS WITH STREPTOKINASE

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and Robert A Good, M D, Minneapolis Minn

The generalized Shwartzman reaction is associated with deposition of homogeneous, eosinophilic material with staining characteristics of fibrinoid Deposits occur within the glomerular capillaries, splenic sinusoids, hepatic veins, and pulmonary arteries, as well as within cardiac valves and beneath endothelium of coronary arteries In the developing renal lesion, this fibrinoid material becomes visible at the inner surface of the glomerular capillaries between 2 and 4 hours following the second intravenous injection of endotoxin and is primary to the bilateral renal cortical necrosis which develops later Prevention of the Shwartzman reaction with heparin and other anticoagulants effectively inhibits development of these fibrinoid lesions, but only when administered at the time of or prior to the provoking injection of endotoxin Heparin administered following the second injection of endotoxin appeared to enhance the lesions

In an effort to correct deposition of fibrinoid material and prevent consequent hemorrhagic necrosis, streptokinase, and streptodornase mixtures were given intravenously to rabbits at a time when it was known that fibrinoid deposits had already been formed. This treatment appeared to reverse the fibrinoid deposition and prevented development of renal necrosis. It was found that this effect is due to streptokinase and will prevent completely the generalized Shwartzman reaction when streptokinase is administered up to 4 hours following the second intravenous injection of endotoxin. Quantities of streptokinase effective in preventing the Shwartzman lesions were found to have no effect on the febrile response development of leukopenia and leukocytosis, inhibition, leukocytic migration, decrease in platelet count, production of heparin precipitable protein, or increase in fibrinogen levels which normally accompany injection of endotoxins. Administration of streptokinase resulted in immediate activation of fibrinolytic capacity of the blood which persisted for periods of more than 3 hours.

Similar results were obtained with the local Shwartzman reaction and the visceral vascular reaction produced by a single intravenous injection of endotoxin together with polyethanol sulfonate.

We conclude from these studies that deposits of fibrinoid material which occur in the course of the generalized Shwartzman reaction are reversible by treatment with streptokinase and that such treatment prevents the progress of the lesions to hemorrhagic necrosis.

25. CHYLOMICRON-LIPOPROTEIN DISTRIBUTION AND THE DYNAMICS OF LIPID TRANSPORT

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(By Invitation), George J. Hamwi, M.D., and J. B. Brown, Ph.D.
(By Invitation), Columbus, Ohio

Although lipids are absorbed from the intestine and transported in serum by a well-defined chylomicron-lipoprotein system, the origin of specific components in this system and their metabolic interrelationships are not understood. Metabolic transformations between different lipoproteins have not been investigated by the usual tracer techniques, since labeled lipids (cholesterol and phospholipid) in one lipoprotein component of the system were shown to exchange, in vitro, with lipids in the other lipoprotein components. In the present investigation, triolein tagged with I^{131} was found suitable to study chylomicron and lipoprotein transformations since the labeled triolein did not exchange, in vitro, between different lipoprotein classes.

The chylomicra and lipoproteins in human serum were partitioned by ultracentrifugal flotation in a density gradient (Oncley and associates). Quantitative lipoprotein distributions were then estimated from cholesterol and phospholipid analyses of each lipoprotein fraction. Three characteristic lipoprotein distribution patterns were observed: (1) Normal; (2) Hyperlipemia in S_f 10-400 lipoproteins; and (3) Hyperlipemia in S_f 3-9 lipoproteins. In patients with a clinical diagnosis of idiopathic hyperlipemia, xanthoma tuberosum, the nephrotic syndrome, and the Kimmelstiel-Wilson syndrome, an elevation in S_f 10-400 lipoproteins occurred. An elevation in S_f 3-9 lipoproteins was found in some patients with a clinical diagnosis of diabetes mellitus, atherosclerosis, and in all patients with myxedema.

In normal individuals, iodinated triolein was absorbed and transported primarily by the chylomicra. The iodinated triglyceride reached its maximum concentration in serum about 4 hours after administration and was cleared from the serum in 24 hours. Labeled triglyceride was not found in either

S_f 3 9 or high density lipoproteins Individuals with an S_f 3 9 hyperlipemia absorbed and transported triolein in the same manner as normal individuals Individuals with an S_f 10 400 hyperlipemia had a distinctly altered pattern Labeled triolein was carried primarily in the S_f 10 400 lipoprotein fraction Furthermore, the triolein was cleared from the serum at a slower rate

These results may be interpreted in at least two ways (1) S_f 10 400 lipoproteins are derived from chylomicra S_f 10 400 hyperlipemia results from a decrease in the rate of clearing for this lipoprotein class, and (2) chylomicra and S_f 10 400 lipoproteins are synthesized independently S_f 10 400 hyperlipemia results from both increased synthesis and decreased clearing in this lipoprotein class

26 CONTROLLED STUDY OF A POLYVALENT ADENOVIRUS INFLUENZA VACCINE

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(Introduced by John E Rebuck, M D)

A controlled study concerning antibody response and tissue tolerance to polyvalent adenovirus influenza vaccine was undertaken in November, 1956

Three groups of approximately 37 individuals each were studied Group I received fluid vaccine containing adenovirus types 3, 4 and 7, Influenza type A (PR8, PR301, and Swine), and Influenza B (Great Lakes and Lee) Group II received a vaccine containing the same antigens which had been absorbed onto aluminum phosphate Group III was given a placebo consisting of balanced salt solution A single booster was administered 16 weeks after primary vaccination Control prevaccination sera were drawn from all subjects at the onset of the program and thereafter at 2 16 (prebooster), and 18 weeks Additional sera were obtained from one third of the subjects in each group at 6, 10, and 14 weeks All sera were tested in tissue culture for neutralizing antibodies against type 3, 4, and 7 adenovirus Influenza hemagglutination inhibition tests were performed on both control and postvaccination sera

The results showed that adenovirus antigen combined in an adenovirus influenza vaccine elicted a satisfactory antibody response The magnitude of the change in antibody titer was similar to that observed by Bell and coworkers when adenovirus antigen alone was employed Aluminum phosphate absorbed vaccine produced superior antibody response when compared to the fluid vaccine

Following a booster dose of polyvalent vaccine, increase in adenovirus antibody titer was noted only if the prebooster titer was fairly low or if the subject showed no antibody at the time of the booster These results also indicated that the antibody titer resulting from vaccination was quite stable over an 18 week period

Influenza hemagglutination inhibition antibody tests yielded an average arithmetical mean fold increase titer for all the contained strains of influenza virus of 0 3 in the control group and 4 5 and 3 9 in the two groups receiving the vaccine

No significant differences in the tissue tolerance were noted with the two types of vaccine

27. A COMPARISON OF THE EFFECTS OF TOLBUTAMIDE AND INSULIN ON PERIPHERAL GLUCOSE UPTAKE IN MAN

James W. Craig, M.D. (By Invitation), Valerie J. Molzahn (By Invitation), William R. Drucker, M.D. (By Invitation), Max Miller, M.D., and Hiram Woodward, Jr. (By Invitation), Cleveland, Ohio

The mechanism of the hypoglycemic action of the sulfonylureas has not been explained satisfactorily. Several workers have compared the metabolic effects of insulin and the sulfonylureas. The results of these investigations have been conflicting, particularly in regard to the effect of the sulfonylureas on peripheral glucose uptake.

In the present study the direct effects of tolbutamide and insulin on glucose uptake in the forearm were compared by injecting the drugs into the brachial artery. Two tests were performed on each of 6 male patients; on one occasion tolbutamide was injected and at another time insulin was used in order to compare the two compounds in the same individual. Blood samples were obtained simultaneously through indwelling needles in the brachial artery and a major antecubital vein. Two-tenths or 0.4 Gm. of sodium tolbutamide (Orinase) or 0.01 unit per Kg. of glucagon-free insulin were injected into the brachial artery and blood samples were drawn at 5 or 10 minute intervals for the next hour. Insulin increased the A-V glucose difference in 5 of the 6 subjects; the mean difference ± s.d. was 0.3 ± 2.0 mg. per 100 ml. before injection and 6.4 ± 3.9 mg. per 100 ml. during the first 30 minutes after injection. Tolbutamide produced no definite increase in A-V glucose difference in any of the 6 subjects during the first 30 minutes after injection; the mean control value was 3.2 ± 2.3 mg. per 100 ml. and the mean value after tolbutamide was 1.3 ± 2.2 mg. per 100 ml. No direct stimulatory effect of tolbutamide on peripheral glucose uptake was demonstrated in contrast to the results with insulin.

In 2 out of 3 cases in which peripheral A-V glucose differences and peripheral arterial-hepatic venous glucose differences were determined simultaneously after injection of 2 Gm. of tolbutamide into a peripheral vein, the fall in arterial blood glucose was accompanied by a decreased splanchnic glucose output without an increase in peripheral A-V difference. The significance of these results in relation to current theories of the mechanism of action of tolbutamide will be discussed.

28. CHLOROTHIAZIDE: A PHYSIOLOGIC CONNECTING LINK

Archer P. Crosley, Jr., M.D., and Robert E. Cullen, M.D. (By Invitation), Madison, Wis.

The purpose of this paper is to demonstrate in acute and chronic studies in 16 patients with renal and/or cardiac disease the manner in which chlorothiazide, a nonmercurial diuretic, serves as a physiologic connecting link between the activity of carbonic anhydrase inhibitors and the mercurial diuretics. Thus, this agent which inhibits carbonic-anhydrase (C-A) in vitro is similar to acetazolamide, *p*-sulfamylbenzoic acid, and dichlorphenamide in its ability to produce significant reductions in glomerular filtration rate ($p = <0.01$) and renal blood flow ($p = <0.001$) while simultaneously causing a significant increase in urinary pH ($p = <0.01$) due to the increased excretion of sodium ($p = <0.001$) and potassium ($p = <0.01$). However, in contrast to C-A inhibitors, this compound, like the mercurial diuretics is capable of producing a significant chloruresis ($p = <0.01$) and diuresis ($p = <0.001$) In a similar fashion, on chronic administration, a hypokalemic hypochloremic alkalosis may be observed rather than the hyperchloremic acidosis which may occur following the use of acetazolamide

Therefore, these results (1) establish a physiologic connecting link between the C A inhibitors and the mercurial diuretics (2) demonstrate the effectiveness of chlorothiazide as a valuable adjunct in the treatment of edematous states and (3) have physiologic significance when related to the renal mechanism involved in the excretion of water and electrolytes

29 THE HEMODYNAMIC RESPONSE TO EXERCISE IN PULMONIC STENOSIS

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The hemodynamic response to exercise has been investigated in 5 women having isolated pulmonic stenosis without clinical evidence of congestive heart failure Data were obtained during right heart catheterization utilizing a double lumen catheter to obtain simultaneous pressures from the right ventricle and pulmonary artery The following parameters were examined at rest and during mild leg exercise (1) cardiac output and stroke volume (2) mean systolic pressure gradient across the stenotic ventricular infundibulum or pulmonary valve, (3) right ventricular end diastolic pressure, (4) pulmonary artery mean pressure

There was a range of 20 to 150 mm of mercury mean systolic pressure gradient between the right ventricle and pulmonary artery at rest with a tendency to rise as much as 15 mm of mercury during exercise In the two patients with the highest mean systolic pressure gradient, the resting cardiac output was reduced However there was an increase in pulmonary blood flow during exercise in all patients ranging from a borderline normal value of 574 ml/100 ml increase in oxygen consumption to a maximum of 1 710 ml Mean pulmonary artery pressure rose in all cases during exercise as much as 10 mm of mercury Right ventricular end diastolic pressure rose to significantly elevated levels in all cases

These data suggest that the observed normal increase in right ventricular output and the increase in mean systolic pressure gradient are achieved at the expense of significant elevation of right ventricular filling pressure in these patients

30 TREATMENT OF HYPERTENSIVE DISEASE IN PREGNANCY

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During the past 6 years approximately 1,200 patients have been treated for so called "toxemia of pregnancy" Forty five per cent of the patients had pre eclampsia and 0 5 per cent had eclampsia but only two patients with these diagnoses received a hypotensive drug In general, we believe the hypertension, if present, is compensatory and it is not treated unless the systolic blood pressure is 200 or higher with proportionate diastolic pressures

Fifty two per cent of the patients had hypertensive disease the majority of some years' duration Approximately 55 patients were hospitalized and placed in an air-conditioned room with no other treatment than either the regular hospital diet or a low-sodium and low-potassium diet. A high percentage of the patients on the above regimen either became normotensive or the blood pressure dropped to such a degree that hypotensive drugs were no longer indicated; this emphasizes the importance of a control period.

Twenty patients who were seen sufficiently early in pregnancy and whose blood pressure persisted for 48 hours or longer despite the above regimen, were thoroughly studied and in each instance the offer was made of therapeutic abortion. These patients were all anxious to have the child if it could be done with reasonable safety. The patients were followed by one man as ambulatory patients and were hospitalized for 5 to 10 days every 4 to 6 weeks.

Seventy per cent of these patients left the hospital with a live baby, approximately the same per cent which Dieckmann has reported previously without the use of hypotensive drugs. To date, hypotensive drugs in the treatment of hypertensive disease in pregnancy have not decreased proteinuria and have not increased fetal survival.

31. EFFECT OF ORAL DIURETICS ON BLOOD SUGAR

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Utilizing both the Somogyi-Nelson and glucose oxidase methods, the effects of acetazolamide (Diamox) and chlormaredrin (Neohydrin) on blood sugar have been investigated. Acetazolamide was found to act as a mild to moderate hypoglycemic agent in over one-half of the subjects tested. In some diabetics it appeared to be more effective than carbutamide. Certain patients develop untoward reactions when placed on this drug. In some, but not all, of these patients the symptoms are due to hypoglycemia. It is thought that this drug should be used with great caution in patients in whom mild hypoglycemia is known to be harmful.

Chlormaredrin reacts with the Somogyi-Nelson color reagent to give erroneously high blood sugar values. The error may be in magnitude of 400 to 500 mg. per cent. The effect may be found in vivo and in vitro. It is maximal within two hours after ingestion of the drug. This may lead to very confusing results when blood sugar studies are being performed on cardiac patients receiving chlormaredrin.

32. CARBOHYDRATE METABOLISM IN PATIENTS UNDERGOING SURGERY WITHOUT ANESTHESIA

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Surgery with anesthesia, or ether anesthesia alone, will cause abnormalities in carbohydrate metabolism. Glucose, pyruvate, and lactate tolerance becomes altered, whereas fructose tolerance remains essentially unchanged following these stresses. It is possible that some of the reported metabolic changes found following surgery could be due to the anesthesia rather than the result of operative trauma.

The present study was designed to determine the effect of surgery without associated anesthesia on carbohydrate metabolism. Intravenous glucose, fructose, and lactate tolerance tests were performed on paraplegic patients operated for repair of decubitus ulcers. The surgical procedures could be carried out in these individuals, who had spinal cord injuries, without the use of anesthesia. Six patients received glucose and fructose tolerance tests on succeeding days before and after surgery. Six additional patients received lactate tolerance tests before, during, and following surgery. The levels of glucose, fructose, pyruvic acid, and lactic acid were determined periodically in venous blood.

The data obtained from these patients operated upon without anesthesia indicate that: (1) a marked abnormality occurs in postoperative glucose tolerance which persists for at least two days; (2) there is no abnormality in the postoperative tolerance for fructose; and (3) a decreased tolerance occurs for lactate during surgery and on the first postoperative day. Similar results were found with patients operated upon with anesthesia. In contrast, the abnormality in glucose tolerance which occurs during anesthesia without surgery disappears within twenty-four hours.

These results have been interpreted as indicating that surgical trauma causes alterations of intermediary metabolism which do not depend on associated anesthesia. Since the alterations of carbohydrate metabolism occur when surgery is carried out below the level of sensation in paraplegic patients, factors in addition to central nervous system stimulation are implicated in the etiology of these changes. It is postulated that the effect of surgical trauma without anesthesia is to alter specific enzymatic processes since the metabolism of glucose and lactate, but not fructose, are altered postoperatively.

33. COMPARATIVE DIAGNOSTIC VALUE OF URINARY COMPOSITION AND TRANSLUMBAR AORTOGRAPHY IN RENAL HYPERTENSION

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Occlusive renal artery disease, an important cause of hypertension, is best demonstrated currently by aortography which, with our technique, is a safe procedure. A recent report suggests that aortography is unnecessary in diagnosing a unilateral arterial lesion since the affected kidney is believed always to excrete a urine of smaller volume and lower sodium concentration. Analyses of ureteral urine are of no help in diagnosing bilateral lesions.

This report concerns a study of separated renal functions in hypertensive patients, some having renal arterial lesions. Urine was collected from each ureter during mannitol loading and Pitressin antidiuresis. Functions studied were glomerular filtration rate (GFR), urine flow (V), urine sodium concentration (U_{Na}), and the excretion fraction of the filtered sodium load (EF_{Na}).

The functions were bilaterally equal in 10 patients with essential hypertension. In 4 patients with (predominantly) unilateral pyelonephritis, GFR and V were less on the more affected side while U_{Na} and EF_{Na} were identical. In 4 with unilateral arterial lesions, GFR and V were depressed on the affected side; in 3 there were associated decreases of U_{Na} and EF_{Na}, but in the fourth these values were bilaterally equal. In two patients with bilateral arterial lesions, differences in GFR occurred between the two sides with corresponding V changes; U_{Na} and EF_{Na} were *higher* on the sides of lower GFR. In two with unilateral arterial and contralateral parenchymal disease, GFR was lower on the sides with parenchymal disease; differences in U_{Na} were insignificant but EF_{Na} were *less* on the sides of the arterial lesions.

The data show a general association between GFR and V in renal disease. However, a relative decrease of U_{Na} is not a consistent characteristic of occlusive arterial lesions. The data emphasize the need for radiologic visualization of the main renal arteries in the diagnosis of unilateral and bilateral occlusive lesions and of combinations of arterial and parenchymal disease since these may unpredictably affect urine volume and composition.

34. VOLUME CHANGES IN THE FOREARM VEINS OF MAN DURING VOLUNTARY HYPERVENTILATION

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(Introduced by James W. Culbertson, M.D.)

Hyperventilation caused an increase in cardiac output and forearm blood flow, a fall in arterial pressure and circulation time, and no change in central venous pressure (Burnum, Hickam, McIntosh). Venous dilatation during this reported arteriolar dilatation would be inconsistent with the increased output and unchanged central pressure. Our study demonstrates and quantitates the forearm venous responses to hyperventilation.

Observations on end-expiratory pCO_2 (pCO_2), minute-volume ventilation, forearm venous distensibility (VD), and dependent forearm venous pressure (VP) were made before, during, and after voluntary hyperventilation in 16 experiments on 12 normal supine men. The venous pressure-volume curve obtained during the plethysmographic measurement of VD expresses the forearm venous volume existing at *any* effective venous pressure (EVP) between 0 and 30 mm. Hg. The naturally occurring venous volume (VV) is the volume coordinate of the point on the curve corresponding to the *measured* VP (= EVP) in the forearm unencumbered by the plethysmograph. VD is designated arbitrarily as the venous volume at an EVP of 30.

During hyperventilation, pCO_2 averaged 22.4 mm. Hg and minute-volume ventilation averaged 31.7 L./min. VD averaged 4.1 c.c./100 c.c. of forearm tissue (range, 3.1 to 5.7) during control periods and fell to 3.3 (range, 1.9 to 4.9) during hyperventilation. VP decreased in 12 and increased in 4 experiments: the average change was a fall of 1.2 mm. Hg. VV fell from an average control value of 2.9 c.c./100 c.c. of forearm tissue (range, 1.5 to 3.5) *to* 1.9 (range, 0.4 to 2.8)—a venous volume reduction of 35 per cent. Recovery from this uniformly observed venoconstriction was incomplete until pCO_2 returned to control levels. The VV decrease observed with room air hyperventilation was prevented partially in 4 of 5 cases by increasing inspired pCO_2 (preventing hypocapnia).

These experiments demonstrate that forearm venous dilatation does not accompany the reported forearm arteriolar dilatation observed during hyperventilation: on the contrary, the forearm veins constrict significantly during this stimulus. This degree of peripheral venoconstriction appears adequate to provide the increased venous return required for the reported increase in cardiac output and is consistent with the unchanged central venous pressure.

35 A DISCREPANCY BETWEEN TYPE OF GONAD AND THE CHROMATIN PATTERN "OVARIES" WITH CHROMATIN NEGATIVE (MALE) SOMATIC CELLS

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E G Bertram, Ph D (By Invitation), Milwaukee Wis

Several different observers have reported the finding of a female chromatin pattern in most cases of apparent males with seminiferous tubule dysgenesis (so-called Klinefelter's syndrome) In some of these even gametogenesis of the male type has been observed This finding appears to represent the first demonstrated example of a complete sex reversal in humans because gonadal sex (when it can be identified) corresponds to the sex chromatin pattern The reverse situation, i e, the finding of ovaries containing follicles and germinal cells in an apparent female with a male type of chromatin pattern has not yet been reported This communication reports such a case the patient differed from most cases of "gonadal agenesis" (Wilkins) in that the patient was not short, had no recognizable congenital anomalies and the urinary FSH was not elevated

The 24-year old slender farmer's wife had primary amenorrhea measured 5 feet, 5½ inches, had sparse sexual hair virtually absent breast development and hypoplastic vaginal mucosa low 17 ketosteroid excretion (2 to 4 mg /24 hr), absent urinary FSH (less than 6 M U 24 hr) hypoplasia of feminine external genitalia and uterus, well developed broad ligaments and Fallopian tubes, "ovaries" (albeit poor quality) containing primordial follicles a few germinal cells, atretic follicles and an increase in fibrocollagenous tissue Cells of skin and buccal mucosa were chromatin negative (male)

The discrepancy between the type of gonad and chromatin pattern as exemplified by this case and by cases of so called Klinefelter's syndrome presents a serious challenge to current theories of sex differentiation and the histologic method of "sexing" We tentatively suggest that if a genetically determined chemical inductor or "evocator" influences the primitive genital ridge to develop into either testis or ovary and if the nature of the gonad can be *opposite* to that of the cellular chromatin pattern then the chromatin pattern (at least under these circumstances of abnormal sexual development) does not accurately reflect the qualitative preponderance of male or female "evocator" Chromatin negativity in our case may not represent "maleness" but merely lack of it and female preponderance

36 LATENT ADENOVIRUS INFECTIONS OF THE HUMAN RESPIRATORY TREE

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(With the Technical Assistance of B Evans and G Gogat)

The presence, frequency, and mechanism of latent virus infections of different parts of the human respiratory tree have been studied in order to understand better the possible role such agents might play in recurrent respiratory illnesses To this end human tonsil, lung, and lymph node tissue removed at surgery have been cultured for prolonged periods of time up to 9 months in a roller drum, observed for cytopathogenic changes, and the supernatant fluid tested for virus at frequent intervals in HeLa and other cells Tissue from

30 tonsils, 9 lungs, and 4 lymph nodes have been successfully cultivated. Adenovirus has been recovered from 15 tonsils and 1 lung after periods of tissue growth averaging 30 days and ranging from 8 to 70 days. No other virus has been clearly identified. Isolations were most frequent in the 6 to 10 age groups. Adenovirus types recovered included four type 1, eleven type 2, and one type 5. The serum from all but two of the individuals from whose tissue virus was recovered contained demonstrable neutralizing and complement-fixing antibodies to the type isolated. In the 2 exceptions there had been a long delay in the emergence of virus from tonsil tissue suggesting a weak antigenic stimulus. In the serum from individuals from whom no virus was isolated antibody was present in some, absent in others.

Factors bearing on the demonstration of virus and its relation to latency and "masking" were studied in detail. Adenovirus could not be recovered directly from the original material itself even after mincing or homogenization. It could not be demonstrated by implanting pieces of the original tissue directly on to HeLa cells. Virus was found under most circumstances only by growing the tissue itself for about a month and continued to be present for periods up to 70 days. Further data have been obtained indicating that the term "masking" is probably not appropriate to this phenomenon, which is largely explicable on a quantitative basis.

37. THE CLEARANCE OF HETEROLOGOUS PROTEIN FROM THE CIRCULATION OF NORMAL AND IMMUNIZED MAN

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The clearance rates of intravenously administered antigenic protein have been extensively studied in normal and immunized animals, but hitherto no such studies have been made in man. Previously only large doses of antigen (mg./Kg. body weight) have been used and relatively little attention has been given to the early phases of protein removal when antigen-antibody union in the circulation might be expected to influence the results. Consequently the events occurring during the early phases of immunologic insult in man have remained obscure.

Streptokinase, a product of hemolytic streptococcal metabolism, is antigenic to man and as a consequence of naturally occurring infections, the population exhibits varying levels of specific circulating antibody toward this substance. Preparations of streptokinase, homogeneous by biophysical examination, but showing traces of impurity on immunochemical analysis were trace-labeled with I^{131} without loss of biochemical activity or detectable change in physical properties.

The patient's total circulating antibody was calculated and clearance studies were made during states of both relative antibody and relative antigen excesses. Streptokinase was removed from the circulation at a rapid exponential rate (average 50 per cent plasma clearance time 18 minutes) when doses less than those required to combine with the total circulating antibody were injected intravenously. Larger doses, greater in amount than those required to neutralize circulating antibody, showed complex clearance curves. Analysis revealed these curves to consist of two exponential component rates. The "fast" exponential component was similar to that found earlier during clearance studies conducted during states of antibody excess (vide supra) and the "slow" component had an average 50 per cent plasma clearance rate of 81 minutes The two rates differed significantly ($p < 0.001$), the "fast" rate being found during antigen clearance in the presence of specific antibody and the "slow" rate in its absence

The mechanisms of antigen removal revealed by this study may be of general biologic importance since the experimental conditions used (a soluble antigen antibody system and a dosage of only a few micrograms antigen per kilogram of body weight) would appear to be analogous to events occurring in human disease states

38 ELECTRONMICROSCOPIC STUDIES OF RENAL BIOPSIES TAKEN FROM NEPHROTIC PATIENTS BEFORE AND AFTER DIURESIS

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Biopsies from the kidneys of 3 healthy and 8 nephrotic individuals were studied by electronmicroscopy, by oil immersion light microscopy using ultra thin sections, and also by standard histologic techniques

Electronmicroscopically, *in healthy human glomeruli, lamina densa* (basement membrane), podocytes (epithelial cells), and endothelial cells were clearly defined The lamina densa was ± 80 mμ thick, homogeneous, structureless, and separated on either side by a clear (± 5 mμ) space from endothelial cytoplasm and podocytic processes (pedicels) Each pedicel was attached separately to the lamina densa The lamina fenestrata (endothelial cytoplasm) completely invested the inner or capillary surface of lamina densa

In one patient, with diffuse thickening of basement membrane by light microscopy, the lamina densa was greatly widened, and split longitudinally The podocytes and their pedicels were normal This anatomic picture is in accord with the concept that proteinuria in the nephrotic syndrome results from increased permeability of a damaged basement membrane

However, in most cases the endothelial cells and lamina densa were completely normal The podocytes were quite abnormal Their pedicels had disappeared, and in their place a continuous layer of cytoplasm invested the lamina densa The podocytic cytoplasm was vacuolated Minute osmiophilic fragments apparently podocytic in origin, were observed lying free in the capsular or Bowman's space Three cases were studied before and after diuresis For example, in one case proteinuria of ± 15 Gm per day decreased to ± 2 Gm per day after diuresis induced by prednisone In this instance a second biopsy taken after diuresis revealed that the podocytes were almost normal, normal discrete pedicels were now seen These observations indicate that the podocyte may play a role relative to the ultrafiltration of plasma, previously attributed by some to the endothelial cells and basement membrane

It has been suggested that the nephrotic syndrome is the result of immune reactions in the glomerulus—localized in the basement membrane Our findings do not support a predominant role of glomerular basement membrane changes in the pathogenesis of these cases of nephrotic syndrome, but rather emphasize the importance of glomerular epithelial cell alterations

39. MECHANISMS OF ACTION OF DIURETICS AS REVEALED BY POTENTIATION STUDIES

Ralph V. Ford, M.D., Houston, Texas

(Introduced by John H. Moyer, M.D.)

Initial experiments in laboratory animals by Pitts (personal communication) suggested that the mechanisms of three groups of diuretics were noncompetitive. Utilizing patients with previous congestive heart failure, we have observed the acute excretory responses of sodium, potassium, chloride, and bicarbonate following the parenteral administration (priming and sustaining doses) of meralluride (Mercuhydrin), acetazoleamide (Diamox), and chlorothiazide (Diuril), separately and sequentially in various combinations.

After a maximum increase in sodium excretion following meralluride injection was observed, the administration of acetazoleamide produced another significant increase (twofold) indicating that two noncompetitive mechanisms for the tubular reabsorption of sodium had been inhibited. At the peak of this second increase, and while meralluride and acetazoleamide were still being infused, the administration of chlorothiazide was observed to produce a third significant increase (twofold) over the previous rate of sodium excretion, suggesting that a third mechanism had been affected.

To further elaborate on the competitive nature of these three mechanisms of diuretic action, the order of administration of the drugs was changed. If acetazoleamide followed the administration of chlorothiazide, no augmentation of sodium excretion was observed. Since at least part of the pharmacodynamics of chlorothiazide involves carbonic anhydrase inhibition, one may conclude that it is capable of acting on this enzyme in a competitive fashion with reference to acetazoleamide. But since chlorothiazide has other pharmacodynamic properties its effect on sodium excretion cannot be blocked by acetazoleamide. If meralluride administration in equipotent doses followed the administration of chlorothiazide, no augmentation of sodium excretion was observed, but the converse is not true. Therefore, chlorothiazide appears to be capable of competing with the mechanism for sodium excretion that is affected by meralluride.

Thus, the data indicate that meralluride shares a mechanism of action with chlorothiazide, that acetazoleamide shares a mechanism with chlorothiazide, and that chlorothiazide can act through a third noncompetitive mechanism.

40. OBSERVATIONS ON THE PATHWAYS OF CHOLESTEROL EXCRETION IN MAN

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(Introduced by F. W. Hoffbauer, M.D.)

The classic concept of the mechanism by which cholesterol is removed from the body was that it is excreted through the intestinal mucosa, converted to coprastanol in the lumen, and eliminated in the feces. More recent experiments with isotopes have been interpreted as favoring the conversion to bile acids as the major pathway of excretion. We have been reluctant to accept this notion because we have been unable to isolate from feces the quantities of bile acids and their derivatives predicted from the rate of cholesterol turnover, and because feces from subjects fed sterol free diets contain sizable amounts of digitonin precipitable material, the source of which is obscure if it does not represent cholesterol excreted from the blood For these reasons, we undertook the present experiments

A healthy man was fed a formula diet of skim milk sugar, and chocolate Twenty microcuries of cholesterol 4 C^{14} were given intravenously Stools were collected daily for isolation and quantitation of the various excretion products of cholesterol, and for comparison of their specific activities with that of the blood cholesterol

The stools were homogenized and extracted exhaustively with boiling ethanol This treatment removed more than 99 per cent of the radioactivity About 70 per cent of the radioactivity was extractable into petroleum ether after hydrolysis with alcoholic KOH Of this, 80 per cent was precipitable with digitonin About 600 mg of digitonin precipitable sterols could be isolated daily, and these had a specific activity equal to that of the blood cholesterol two days earlier Only 15 per cent of the daily excretion of radio activity was recovered in the bile acid fraction, two thirds of which was isolated as deoxycholic acid

We feel that these results, together with other evidence (e g, J Clin Invest 36 898, 1957), demand that further consideration be given to the possibility that the older views on the mechanism of cholesterol excretion were correct

41 A COMPARISON OF THE USEFULNESS OF I^{131} TRIOLEIN, RISA, AND FAT AND NITROGEN BALANCE TECHNIQUES IN THE DIAGNOSIS OF MALABSORPTION

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Triolein and human albumin (RISA), tagged with I^{131}, are now widely used as tools for the diagnosis of malabsorption syndromes This is based on the demonstration by several laboratories that differences in absorption of these substances can be used to separate normal from grossly abnormal patients The efficiency and reliability of these methods as compared with traditional fat and nitrogen balance studies have not yet been clearly demonstrated

The present study compares the results of 5 day fat and nitrogen balances with the results of fecal, urinary, and blood radioactivity determinations following administration of I^{131} triolein or RISA, and with simultaneous vitamin A and glucose tolerance curves in over 50 subjects Diseases studied include nontropical sprue, Whipple's disease, pancreatic insufficiency, post gastrectomy steatorrhea, regional enteritis, and ulcerative colitis In general the correlation between increased fecal radioactivity and excessive fecal fat and nitrogen was good Sensitivity in demonstrating abnormality was greater with triolein than with RISA A small but important group of patients showed striking lack of correlation between radioactivity and chemical determinations Lack of correlation was greater and more frequent when blood or urinary radioactivity was compared. It is concluded that fecal radioactivity determinations are more useful than blood or urine determinations and that none of these show complete agreement with chemical balance studies.

42. GONADAL AND ADRENAL STEROID-INDUCED CHANGES IN THE CHOLESTEROL AND PHOSPHOLIPID CONTENT OF SERUM LIPOPROTEIN FRACTIONS ISOLATED BY SIMULTANEOUS CENTRIFUGATION AT FOUR SOLVENT DENSITIES FOLLOWED BY QUICK-FREEZING AND TUBE-SAWING

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A simple technique for separation of serum lipids according to the specific gravity of the lipoprotein complex is described. Serum aliquots are spun at 40,000 r.p.m. (144,700 × g) for 22 hours at the following densities: 1.006, 1.019, 1.063, and 1.21 g per ml. The tubes are removed from the rotor without agitation and "quick-frozen" in a solution of dry ice and acetone. The plastic tube and its solid contents are sawed through in the clear zone beneath the supernatant fraction. The infranatant and supernatant fractions are then placed in funnels in volumetric flasks, allowed to melt and made up to standard volume with methanol-methylal. Lipid analyses are then carried out on the filtrates.

Five subjects were given peroral methyltestosterone, one equilin sulfate, three prednisone, and one cortisone acetate.

The cholesterol and phospholipid content of native serum and of each density-fraction was determined, and the percentage distribution of these lipids in each fraction calculated.

Equilin sulfate, cortisone, and prednisone produced absolute and relative increases in the amount of lipid in the highest density $> 1.063 < 1.21$ g per ml. ("alpha") fraction, while methyltestosterone reduced the lipid content of this fraction.

The lipid content of the $> 1.019 < 1.063$ ("beta") lipoprotein fraction showed a less consistent response. Equilin sulfate and cortisone reduced the cholesterol and phospholipid content of the beta lipoprotein fraction, both relatively and absolutely. Methyltestosterone administration led to increases in beta lipoprotein cholesterol and phospholipid with one exception when no change was noted.

Increased beta lipoprotein lipid following prednisone administration was noted only when marked increase in the native serum cholesterol and lipid phosphorus occurred. When no increase in native serum lipid concentration was noted following prednisone administration, beta lipoprotein lipid concentration fell, absolutely and percentagewise.

The cholesterol and phospholipid content of the > 1.006, < 1.019, and < 1.006 fractions either fell or underwent no change during equilin sulfate or adrenocorticosteroid administration, whether or not native serum lipid concentrations increased; the response following methyltestosterone was slight and inconsistent.

The effects of adrenocorticosteroids on serum lipids and lipoproteins do not appear to be a consequence of thyroid suppression.

43. STUDIES IN THE PATHOGENESIS OF LUPUS ERYTHEMATOSUS: EXPERIMENTAL PRODUCTION OF HEMATOXYPHIL BODIES IN THE KIDNEY

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(INTRODUCED BY F. J. HECK, M.D.)

Observations under phase contrast microscopy of unstained preparations containing L.E. cells and L.E. bodies disclosed that these structures, in addition to being relatively rigid and "sticky," were often larger than normal formed elements of the circulating blood. This suggested that obstruction to small blood vessels could develop if such structures should circulate in the blood stream.

These observations, combined with the known histochemical identification of in vitro L.E. bodies with lupus tissue "hematoxyphil" bodies and the unique pathognomonic significance of the latter, prompted animal experimentation.

Suspensions of L.E. cells and L.E. bodies were injected into the aortas of rabbits and guinea pigs. The kidneys, removed and fixed from 15 minutes to 4 hours after injection, showed an abundance of structures in the glomerular capillaries which by contour, texture, and staining reactions (purple on H and E stain; Feulgen positive) seemed characteristic of the hematoxyphil, or "hematoxylin," bodies found in the lupus kidney. Control animals were negative.

44. THE EFFECT OF BLOCKING DRUGS ON THE VASCULAR REACTION TO ENDOTOXIN IN CATS

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Histamine, 5-hydroxytryptamine (5-HT) and epinephrine have been suggested as mediators of the vascular responses to bacterial endotoxin. Chlorpromazine and dibenzyline, which block the effects of one or more of these drugs, have been shown to protect mice against the lethal effects of endotoxin. The present study concerns the effect of similar blocking drugs on the early vascular responses to endotoxin.

Carotid (CAP) and pulmonary (PAP) arterial pressures were measured in the open chested cat under pentobarbital anesthesia. The administration of endotoxin to the cat characteristically causes a PAP rise and a CAP fall. Because antihistaminics and dibenamine did not block the vascular effects of large doses (0.1 to 1.0 mg./Kg.) of histamine and 5-HT, a dose of endotoxin was chosen which did not produce an overwhelming effect.

In 15 control cats, 0.05 mg./Kg. of endotoxin caused a CAP fall of 45 per cent (± 9 s.e.) and a PAP rise of 73 per cent (± 17 s.e.). The combination of dibenamine (15 mg./Kg.) and pyrilamine (5 mg./Kg.) afforded almost complete protection in 5 cats: the CAP rose 3 per cent (± 7 s.e.) and the PAP rose 27 per cent (± 10 s.e.). In 5 trials of each agent alone there was only partial protection. The combination of LSD (0.5 mg./Kg.), with no adrenolytic activity, and pyrilamine gave fair protection in 4 cats: the average CAP fall was 12 per cent and the average PAP rise was 15 per cent. LSD alone mitigated the fall in systemic pressure, but did not alter the PAP rise. The effect of large doses of endotoxin (5 mg./Kg.) was not appreciably lessened by these drugs.

These results are consistent with the possibility that histamine and 5-HT are active during the early phase of the endotoxin reaction. It does not follow that the blocking agents used would be of therapeutic use, especially during the late phases of a severe endotoxin reaction.

45. SOME OBSERVATIONS ON THE VARIABLE FATE OF BILIRUBIN DEPENDING ON CONJUGATION AND OTHER FACTORS

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Earlier studies had shown that crystalline bilirubin given per duodenal tube in patients with complete biliary obstruction was largely unaccounted for as urobilinogen in the feces. In the present study intraduodenal administration of N^{15} crystalline bilirubin in 3 cases of carcinomatous biliary obstruction and in a normal subject was followed by recovery of less than 5 per cent as fecal urobilinogen and no increase of mesobilifuscin (dipyrrylmethene group). Crystalline bilirubin given intravenously in a normal subject was largely accounted for (79 per cent) as urobilinogen, with minor amounts of mesobilifuscin and bilirubin. The administration of a crude bilirubin glucuronide per duodenal tube in a normal subject was entirely accounted for as fecal urobilinogen and mesobilifuscin, in the normal ratio. Substantial increases in fecal urobilinogen were noted in similar experiments in two patients with complete biliary obstruction.

A remarkable contrast to the normal handling of bilirubin was observed in a patient with refractory anemia. The Cr^{51} red cell survival measurement was normal, but the fecal urobilinogen was regularly very small, averaging but 22 per cent of the anticipated value. Here there was no significant increase of fecal bilirubin, urobilinogen, or dipyrrylmethene following administration of bilirubin glucuronide intraduodenally or crystalline bilirubin intravenously. Yet when crystalline mesobilirubinogen (*i*-urobilinogen) was given intraduodenally, the increase of fecal urobilinogen corresponded with the amount administered. These observations suggest that in this patient there is an aberrant fate of bilirubin, but whether in the intestinal tract is not clear. That it is not due to failure of conjugation of bilirubin by the liver was evidenced by the usual prompt direct diazo reaction of the bilirubin in the duodenal contents.

The present studies thus indicate that while conjugation of bilirubin is important or even essential to its normal conversion to urobilinogen by the fecal flora, there may exist significant deviations of the ordinary pathway based on other mechanisms which are not yet clear. Whether these are fundamentally related to disease states, such as anemia, also remains to be determined.

46. BLOOD CHOLESTEROL LOWERING EFFECT OF ESSENTIAL FATTY ACID SUPPLEMENTS IN DIABETIC PATIENTS

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Preoccupation with atherosclerosis as an important human disease accounts for the widespread interest in cholesterol and a variety of methods that have been devised to alter blood cholesterol levels. Of these methods, one of the most interesting has been the addition of essential fatty acid supplements (EFA) to the diet. Special interest in diabetic subjects has resulted from (1) the well-recognized accelerated rate of development of atherosclerosis in diabetics, (2) the report of Holman that diabetic animals have an EFA requirement significantly higher than that of normal controls, and (3) the tendency for many diabetics to have elevated blood cholesterol levels.

Fourteen severe diabetic patients of both sexes, ranging in age from 19 to 54, have been studied over a period of 7 months All subjects are suffering from diabetic vascular complications of the Kimmelstiel Wilson type and many have severe loss of vision EFA has been administered in the form of safflower oil in a dose of 30 ml morning and evening accompanied by an isocaloric reduction in the fat of their diabetic diets Measurements have been made serially of total, free and esterified cholesterol, total content, and iodine number of circulating plasma fat Careful clinical observations have accompanied these laboratory studies

Blood cholesterol levels have been consistently and significantly lowered in all subjects No significant change in the free/ester ratio has been noted Total circulating blood lipids have risen progressively in most instances and no consistent change (rise) in iodine number of these fats has occurred All patients have been improved clinically No spontaneous retinal hemorrhages have occurred in any patient since EFA supplements were started and tourniquet tests initially strongly positive, have become less positive and in a few instances, negative Insulin requirements have tended to decrease slightly The possible significance of these observations will be discussed

47 RETROGRADE AORTIC CATHETERIZATION IN AORTIC STENOSIS

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The precise determination of various degrees of aortic stenosis requires direct measurement of the pressure gradient across the valve It seemed technically advantageous particularly in infants and children, to measure this gradient by retrograde catheterization of the aorta and left ventricle from the right brachial artery This procedure was performed successfully, without morbidity and mortality in 15 cases of aortic stenosis ranging in age from 10 weeks to 22 years From this approach it was possible to explore both ascending and descending aorta, which proved the pressure, location and degree of associated coarctation in 3 cases In entering the left ventricle constant care with fluoroscopic visualization and simultaneous pressure monitoring was exerted to first outline the exact position of the valve and then to pass it without traumatizing the coronary arteries Ventricular irritability was notably absent as long as catheterization of this chamber was done with care

In addition to the quantitative physiologic information provided this technique has established by electrocardiographic correlation a foundation for the more accurate clinical determination of the presence degree, and hemodynamic type of left ventricular enlargement In combination with right heart catheterization it has also proved to be of importance in detecting and evaluating the presence of combined valve lesions such as pulmonary and aortic or mitral and aortic stenosis, particularly when other signs such as those indicative of combined cardiac chamber enlargement may be equivocal or absent

48 A PITUITARY PROSTATE RELATIONSHIP NOT MEDIATED BY THE TESTIS AS REVEALED BY STUDIES WITH TESTOSTERONE 4-C^{14} IN THE MALE RAT

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(INTRODUCED BY RICHARD L LANDAU, M D)

After the subcutaneous administration of a physiologic dose of testosterone 4 C^{14} to intact, castrated, and hypophysectomized castrated, pubescent male rats, greater concentrations of radioactivity were detected in the prostate and other male accessory glands than in nonsex-linked tissues. The evidence gathered suggests that the radioactivity detected in the prostate represented the androgen or a rather immediate metabolic thereof.

In intact rats and in rats castrated 2 or 3 days the prostatic concentration of radioactivity averaged 2.5 to 3.0 times that in tissues regarded as "androgen independent" (e.g., tongue). The radioactive isotope could not be removed by perfusion with normal saline. Cannulation of the bile ducts to eliminate hepatic metabolites and intestinal degradation products of testosterone did not diminish the avidity of prostatic tissue for the isotope. The injection of cholesterol-4-C^{14} as a control steroid did not yield similar concentrations of tissue radioactivity.

Hypophysectomy 3 days prior to the administration of testosterone-4-C^{14} lessened the attraction of the prostate for the androgen derived isotope. The mean concentration of radioactivity in the prostates of 2-day castrated animals was 14.7 disintegrations per minute per 10 mg. wet weight, whereas the mean concentration in prostates of hypophysectomized castrated animals was 9.9 disintegrations per minute per 10 mg. wet weight. A comparison of the ventral prostate/tongue ratios of radioactivity (a device employed to cancel the effects of variations in the concentration of circulating isotope on its localization in both prostate and "androgen-independent" tissues, as well as to emphasize the "specific" localization in prostate) delineated the two groups of animals even more sharply. The mean ventral prostate/tongue ratio in castrates was 2.87; in hypophysectomized castrated rats, 1.62 (p value < 0.001).

These data suggest that the localization of androgen by male accessory tissues may be an important facet in the endocrine control of their growth and metabolism. The impairment of this localizing process in hypophysectomized castrated rats points to a mechanism by which the pituitary could influence the prostate by means other than stimulation of the testis.

49. INHIBITORY EFFECTS OF STEROIDS ON THE GROWTH OF UTERINE FIBROBLASTS: THE ISOLATION OF STEROID-RESISTANT VARIANTS

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(Introduced by R. F. Parker, M.D.)

The effect of steroids on the proliferation of mammalian cells cultured in vitro has been investigated. Uterine fibroblasts, strain U12-79, were propagated in a defined medium supplemented with 5 per cent dialyzed horse serum (medium 79). To examine the effects of steroids on growth, U12-79 was propagated serially for periods of 3 weeks (3 subcultures) in medium 79 containing a single steroid in concentrations ranging from 0.1 to 25 μg per milliliter. Cells exposed to corticosterone, desoxycorticosterone, estradiol, estriol, hydrocortisone, progesterone, or testosterone at a concentration of 25 μg degenerated over a period of 2 to 7 days. Hydrocortisone and corticosterone inhibited growth at concentrations as low as 0.1 μg per milliliter whereas the remaining steroids reduced proliferation only at levels of 5 μg per milliliter. Inhibition of growth in the presence of steroids was accompanied by cytologic changes. In view of the fact that a population of U12 cells has been shown to contain nutritional variants (Swim, H. E., and Parker, R. F.: Fed. Proc. 16:

435, 1957) the possibility that steroid resistant variants were also present was investigated Cells were treated with either hydrocortisone or testosterone at a concentration of 25 μg per milliliter Under these conditions, the cells gradually degenerated until it appeared that all of the cells were involved The regular schedule of fluid replacements was continued and subsequently a few healthy cells were noted in the cultures and their progeny continue to proliferate at essentially the same rate in the presence of steroid as that of the U12 79 strain in medium 79 Previous experiments (Sweat, M L, Grosser, B I, Nabors, C J, and Swim, H E Fed Proc 16 258, 1957) indicate that hydrocortisone is metabolized by strain U12 79 Studies are now under way to determine whether the metabolism of hydrocortisone by the resistant variant differs from that of the sensitive U12 79 strain

50 THE STIMULATORY EFFECT OF "ANEMIC PLASMA" IN CONGENITAL HYPOPLASTIC ANEMIA

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Congenital hypoplastic anemia is a chronic anemia appearing in the first few months of life, characterized by a *normochromic anemia*, persistently low reticulocyte count, and marrow hypoplasia confined to the erythrocytic series It is usually refractory to therapy other than repeated transfusions Our studies of the hormonal regulation of erythropoiesis led us to consider the possibility that a deficiency of an essential plasma factor might be responsible for erythroid hypoplasia in this disease This hypothesis was tested by observing the effects of "anemic plasma" in two patients with congenital hypoplastic anemia "Anemic" rather than normal plasma was used because we demonstrated previously the increased erythropoietic stimulating properties of such plasma Donors of the same blood type as the patients were bled until their hematocrit values reached 25 per cent "Anemic plasma" was then harvested daily, the packed cells being returned to the donors to prevent further fall in their hematocrits

Daily infusions of approximately 300 ml of "anemic plasma" were given to two patients with congenital hypoplastic anemia After 5 infusions, the reticulocyte count exceeded any values recorded previously for either patient, rising to 4 0 per cent after 8 infusions in one patient and 3 4 per cent after 6 infusions in the other Iliac marrow aspirations were performed prior to the onset and again upon termination of the course of the infusions In the first patient, who responded with a transient rise in hemoglobin as well as a reticulocytosis, the marrow showed increased numbers of proerythroblasts and all stages of normoblasts In the second patient, no rise in hemoglobin accompanied the reticulocytosis, and marrow changes were less striking although early normoblasts were increased

We conclude that some factor present in "anemic plasma" is capable of stimulating erythropoiesis in congenital hypoplastic anemia This finding suggests congenital hypoplastic anemia is the result of a congenitally determined deficiency of some essential plasma factor Current investigation is directed toward elucidating the nature of this factor and its possible relationship to erythropoietin, the erythrocyte stimulating hormone

51. EFFECT OF CHANGE IN BLOOD FLOW RATE UPON RENAL VASCULAR RESISTANCE

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(Introduced by Craig W. Borden, M.D.)

It has long been known that renal vascular resistance increases progressively as arterial pressure is raised in the range 80 to 200 mm. Hg. This accounts for the unique capability of the kidney to keep its blood flow rate constant despite large changes in arterial pressure. The response has been variously ascribed to active vasoconstriction, passive vasoconstriction, absolute blood viscosity increase, and dynamic blood viscosity increase. In an attempt to define the mechanism responsible for the resistance increase, the effect of changing flow rate in the range 25 to 180 ml./min. upon renal vascular resistance was studied in 82 pentobarbitalized laparotomized dogs. Flow rate was varied by interposing a blood pump in the right renal artery. On the average, resistance progressively decreased when blood flow rate was raised from 25 to 75 ml./min. and progressively increased when raised from 75 to 180 ml./min. However, intraindividual and interindividual comparisons showed the onset of the resistance increase to be flow rate independent but pressure dependent. The onset always occurred at mean arterial pressures between 50 and 100 mm. Hg regardless of the flow rate. This relationship between resistance and pressure was unchanged by extrinsic nerve section, destruction of circulating catecholamines, local sympathetic nerve blockade, local parasympathetic nerve potentiation, increased CO_2 tension, and $[H^+]$ and decapsulation. The resistance increase was abolished and replaced by a progressive resistance decrease during blood perfusion when dextran was intermittently used as the perfusate. The resistance increase was not observed in dextran perfused kidneys and in blood perfused dead kidneys. Elevation of arterial pressure by increasing blood flow rate failed to significantly increase lymph flow rate from cannulated hilar lymph vessels. Winton, Gottschalk, Swann, and Miles have separately shown that elevation of renal artery pressure is accompanied by no or relatively little change in interstitial pressure. These observations indicate that the resistance increase results from active vasoconstriction. The vasoconstriction appears to be caused by some effect of an increase in arterial transmural pressure acting directly upon vascular smooth muscle.

52. THE EFFECT OF ANTIBIOTIC COMBINATIONS UPON STAPHYLOCOCCI, WITH SPECIAL REFERENCE TO OLEANDOMYCIN

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In a previous report we demonstrated the activity of 12 antibiotics in 66 paired combinations against 30 hospital strains of *Staphylococcus aureus.* Activity was measured by broth dilution with subcultures and by paper strip-agar diffusion with replica plating. Addition of antibacterial activities of antibiotics was frequent but true synergism and antagonism were rare with these rather resistant bacterial strains.

Recent claims for synergism of action with delay in emergence of resistance prompted studies with the new antibiotic oleandomycin, in combination with various other antibiotics

Though rather resistant to penicillin and tetracycline, most of the 30 strains of staphylococci were inhibited in broth by a smaller concentration of Sigmamycin (oleandomycin ⅓ plus tetracycline ⅔) AP (oleandomycin ⅓ plus penicillin G ⅔) and APG (AP ⅔ plus penicillin G ⅓) than by oleandomycin, tetracycline, or penicillin These antibiotic combinations also showed addition of growth inhibition on agar plates when tested individually and with bacitracin chloramphenicol, erythromycin, novobiocin neomycin, and vancomycin Antagonism was exceedingly rare and true synergism was never observed on agar plates Oleandomycin was less active than erythromycin

Contrary to early reports, oleandomycin combinations proved to show little addition of antibacterial activity against resistant bacterial strains

A few strains of susceptible staphylococci were made resistant to individual and paired antibiotics by repeated exposure to doubling concentrations in broth Resistance developed rapidly and to high concentrations with the single antibiotics streptomycin, erythromycin, oleandomycin, and vancomycin Resistance to penicillin and to neomycin developed slowly and to relatively low concentrations with most bacterial strains, though a few rapidly became highly resistant Mixtures of equal parts of these antibiotics in pairs were also used Vancomycin with oleandomycin or erythromycin did not retard resistance Streptomycin slowed moderately the rate of resistance to both oleandomycin and erythromycin Contrary to expectation, oleandomycin markedly slowed the rate of resistance to the closely related antibiotic, erythromycin

Antibiotic combinations are useful in staphylococcal infections, especially if antibiotic susceptibility tests are lacking Addition of antibacterial activities may be helpful and emergence of resistant strains may be retarded by a proper selection of antibiotics Mixtures of similar antibiotics may prove more useful than has been apparent hitherto

53 CIRCULATORY DYNAMICS IN ACUTE GLOMERULONEPHRITIS

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Although heart failure is the most common cause of death in the initial stage of acute glomerulonephritis, its pathophysiology is poorly defined To determine the circulatory abnormalities present in this disease, hemodynamic studies, employing the technique of cardiac catheterization, have been carried out in 6 patients with typical clinical and laboratory findings of acute glomerulonephritis of a few days' duration Patients were studied in the resting state

At the time of study, all patients had elevated blood pressures, 5 were edematous, 2 had pulmonary congestion In 5 of the subjects, including both patients with pulmonary congestion the arm to tongue circulation time was within normal limits

In 2 patients oxygen consumption was slightly elevated, but was normal in the remaining 4 The arteriovenous oxygen difference ranged from 25 7 to 42 5 ml /L with a mean of 31 7 ± 2 6 (s e m) The cardiac index ranged from 4 43 to 7 08 L /min /M^2 with a mean of 5 18 ± 0 40, a value significantly above the normal

In all patients the pulmonary "capillary" pressure was elevated, ranging from 14 to 39 mm. Hg with a mean of 23.3 ± 3.7, indicating a high left ventricular filling pressure. The pulmonary artery and right ventricular systolic pressures were abnormally high in each and right ventricular filling pressure was significantly increased in 4 of the 6 patients.

Although all patients were hypertensive with brachial artery mean pressures ranging from 136 to 182 mm. Hg, the calculated total peripheral resistance was within normal limits (range 1,100 to 1,502 dynes/sec./cm.$^{-5}$, mean 1,303 ± 126), indicating that the hypertension in this high output state is independent of any significant reduction in the total cross-sectional area of the peripheral vascular bed. Total left ventricular and left ventricular stroke work were markedly elevated. Two patients studied after diuresis had occurred showed entirely normal circulatory dynamics.

In conclusion, in the edematous stage of acute glomerulonephritis there is a high cardiac output with associated increase in ventricular filling pressures suggesting that these patients have a high output type of heart failure.

54. CLINICAL AND METABOLIC CHANGES INDUCED BY GLUCAGON IN PATIENTS WITH RHEUMATOID ARTHRITIS

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In our laboratories we have been studying the effects of glucagon on carbohydrate and protein metabolism. One of the most striking effects of its administration is the lowering of amino nitrogen in the blood of human subjects and experimental animals. The change in amino nitrogen of the blood is greater than that of glucose. The effect of glucagon on protein metabolism does not appear to be mediated through the adrenal glands, since in adrenalectomized rats the fall in amino nitrogen is of the same order as in the intact animal but is not as sustained.

Salter and Best have also demonstrated that glucagon has a protein catabolic effect, and on this basis they administered glucagon to patients with rheumatoid arthritis. These investigators observed that an anti-inflammatory effect accompanied the protein catabolic action.

With hope of throwing more light on the metabolic mechanisms concerned, 12.5 mg. of crystalline glucagon was given intravenously over a period of 10 hours to 3 patients with rheumatoid arthritis. Each patient received infusions for 3 consecutive days. Of the blood constituents examined, the decrease in amino nitrogen was of greatest magnitude, greater than that of rise in glucose. In the urine there was an increased excretion of nitrogen, phosphate, 17-OH corticoids, and uric acid during the infusion period with variable appearance of ketone bodies. There was minimal glycosuria.

Subjectively, all patients volunteered that there was diminished pain, increased joint motion, and a continued feeling of well-being for periods up to 7 days after the discontinuance of therapy. One patient whose disease was in the acute phase also demonstrated decreased redness of joints. Untoward symptoms were limited to nausea and loss of appetite which occurred throughout the period of infusion.

Because of the nausea and short duration of action, glucagon in its present form appears to have no practical value in the treatment of arthritis. However, it is possible that further experimental study may not only shed light on the basic mechanism of inflammation but also increase our knowledge of protein, carbohydrate, and energy metabolism.

55 PROTEIN REQUIREMENTS IN CHRONIC RENAL INSUFFICIENT PATIENTS

A Study of the Nitrogen Minimum

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The protein requirement for nitrogen equilibrium was estimated from nitrogen balance studies on five chronic renal insufficient patients and one healthy subject. The minimum nitrogen requirement for nitrogen balance in the renal insufficient patients exceeded that of a healthy subject.

The increase in protein requirement for nitrogen balance seemed to be greatest in patients with the most advanced renal insufficiency. Whether this was due to a generally reduced efficiency in protein utilization or to increased requirements for some particular amino acids is not known.

The addition of glycine and a mixture of both D and L forms of the 8 essential amino acids according to Rose resulted in nitrogen loss out of proportion to the increased nitrogen intake; supplementation with L methionine resulted in a nitrogen loss increased in proportion to the increase in nitrogen intake and no protein sparing effect could be demonstrated. The addition of a mixture of L amino acids composed of 75 per cent leucine, 15 per cent isoleucine, 8 per cent methionine, and 3 per cent phenylalanine resulted in a modest nitrogen sparing effect in one patient and no effect in two others.

56 THE INTERPRETATION OF ELECTROLYTE CONCENTRATIONS OBTAINED DURING INTRAVENOUS ELECTROLYTE INFUSIONS

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(Introduced by Sheldon S. Waldstein, M.D.)

In the management of patients with electrolyte abnormalities, an intravenous repair solution is often started before blood samples are obtained for analysis. The significance of the results of these subsequent electrolyte determinations is therefore questioned. This experiment was designed to study this situation in normal subjects preliminary to study of patients with electrolyte abnormalities.

Five hundred milliliters of a solution containing 155 mEq./L. of sodium chloride and 40 mEq./L. of potassium chloride were administered intravenously to each of 20 normal, fasting adults. This was given at a constant rate in exactly 2 hours. Blood was obtained from the opposite extremity prior to and 30, 60, and 120 minutes after the start of the infusion. The microhematocrit and serum sodium, potassium, and chloride concentrations were determined in each blood specimen.

The data were submitted to an analysis of variance. The following changes were found to be statistically significant at the 5 per cent level: a decrease in the hematocrit and an increase in the serum potassium which persisted during the entire infusion, and an increase in the serum chloride concentration 1 and 2 hours following the start of the infusion. There was no significant change in the serum sodium concentration.

The maximal variations of the means were: hematocrit, 3 per cent; serum potassium, 0.38 mEq./L.; and serum chloride, 1.9 mEq./L. If true in abnormal patients, as well, variations of this degree would be unlikely to alter the diagnostic appraisal. This demonstrates very rapid and nearly complete mixing of infused electrolytes in normal patients.

It is concluded that intravenous administration of sodium and potassium chloride at the concentrations and rate described to normal adults does not alter significantly the hematocrit or serum sodium, potassium, and chloride concentrations in blood obtained during the course of the infusion.

57. A CHROMATOGRAPHIC STUDY OF THE CHEMICAL STRUCTURE OF HUMAN SERUM LIPIDS

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(Introduced by Irvine H. Page, M.D.)

Adsorption chromatography with silicic acid is a valuable method for separating the components of many complex lipid mixtures. Recent modifications of this method involving the special selection and preparation of adsorbent and the use of gradient elution render it particularly applicable to the separation and chemical characterization of the components of human serum lipids. Preliminary studies with other adsorbents indicate that such chromatographic separations may be accomplished by a rapid and automatic means.

These techniques demonstrate the existence of the following classes of lipids in the serum: hydrocarbons (carotene), cholesterol esters, triglycerides, nonesterified fatty acids, cholesterol, and phospholipids. The phospholipids are further separable into three substances corresponding to: cephalins, lecithin, and sphingomyelin. In a large number of normal and abnormal sera, no diglycerides or monoglycerides can be found; but, during the heparin-induced clearing of lipemic serum, small amounts of these substances are present.

The complete separation and quantitative recovery of cholesterol esters, triglycerides, and phospholipids enable the isolation of the fatty acids present in each of these groups. The further analysis of these fatty acids by gas-liquid chromatography completes the chemical definition of the serum lipids.

Previous studies have shown that the formula feeding of single, well-defined fats to human subjects produces predictable alterations in the concentration of the serum lipid classes. The complete analysis described above shows striking variation of the fatty acid composition of each lipid class in response to such dietary manipulations. This is most marked in the triglyceride fatty acids, but the cholesterol esters and phospholipids also show distinct changes. Thus, the feeding of a corn oil formula following a butter formula more than doubled the linoleic acid content of the cholesterol esters and phospholipids, but the amount of linoleic acid in the triglycerides was increased *eleven* fold.

58. APPARENT STIMULATION OF THYROID ACTIVITY IN MEN FED AN UNUSUAL DIET

Robert E. Hodges, M.D., and Titus C. Evans, Ph.D., Iowa City, Iowa

(Introduced by Henry E. Hamilton, M.D.)

A group of 6 healthy young men was fed a formula composed of purified casein, corn oil, carbohydrates, vitamins, and minerals. This strange diet has been used by us in previous studies of pantothenic acid deficiency. In this experiment the men were divided into 3 pairs. All 6 were fed the basic formula for 12 weeks, but 2 men serving as controls received pantothenic acid. The other 4 men were deprived of this vitmain, and 2 of these were given an antimetabolite of pantothenic acid.

Thyroid function tests were performed to ascertain any possible aberrations from normal, although none were expected. The responses, while surprising, were unrelated to pantothenic acid or its antagonist, since they were similar in all 6 men.

The protein-bound iodine values which initially averaged 5.2 gamma per 100 ml. of blood, began to rise by the fourth week, and averaged 11.8 by the *sixth week of this diet. At the same time the uptake of radioactive iodine* began to rise. Initially the 24-hour uptake averaged 13 per cent, but this increased steadily to 32 per cent by the end of the study

Clinically the men remained euthyroid. Their thyroid glands did not enlarge and their temperature, pulse, and body weight remained constant.

It became apparent that here was a phenomenon not readily explained by our concepts of thyroid metabolism. The only other significant biochemical change was a prompt decrease in the serum cholesterol from average values of 215 to 134 mg. per cent. This is a normal response to a diet containing highly unsaturated fats.

One further consideration arose. The formula was assayed for its content of iodine and found to be low (less than 0.2 mg. per day). Iodine deficiency is *known to produce goiter and to increase the radioiodine uptake temporarily, but* would the protein-bound iodine also rise? We determined the ratio of bound I^{131} in each man and found it to be normal.

We have considered several possible explanations for this effect but none seems satisfactory. Could unsaturated fats alter the transport of iodine? Perhaps our future studies will reveal whether this is fact or artifact.

59. A SURVEY OF THE RELIABILITY OF THE DISK TEST FOR ANTIBIOTIC SENSITIVITY IN NINE TEACHING HOSPITAL LABORATORIES

R. V. Hoffman, M.D. (By Invitation), G. G. Jackson, M.D., and M. P. Turner, M.S. (By Invitation), Chicago, Ill.

Commercial paper disks for determining bacterial sensitivity to antibiotics are in common use. Their results have considerable influence upon physicians and the treatment of patients. Several studies from individual laboratories have supported or challenged the validity of the test. In the present investigation standard strains of five different bacterial genera were tested by the routine disk method in the laboratories of nine teaching hospitals and by two modifications of the tube dilution method. Major discrepancies occurred in the results from different laboratories in up to 25 per cent of the tests and for individual laboratories in the relation of the results to those obtained by the tube dilution method. The disk test usually showed greater sensitivity than the corresponding tube dilution test. The finding of resistance was more reliable. Over-all, 21 per cent of strains reported as sensitive and 53 per cent of those found moderately sensitive by the disk test were not inhibited by 10 μg/ml. while 11 per cent of strains reported as resistant and 31 per cent reported as moderately resistant were susceptible to this concentration or less. There were marked differences in the correlation of the results for different antibiotics. False sensitivity was common with streptomycin, chloramphenicol, and polymyxin, whereas false resistance was a more common finding with Aureomycin and tetracycline disks. Important differences also were observed among the bacterial species.

The data illustrate the limited reliability of depending upon the results of routine sensitivity tests in the selection of an antibiotic for therapy. The discussion will include the areas of confidence and independability in the clinical interpretation of the tests.

60. A CRITICAL STUDY OF BALL-TYPE EAR PROTECTORS

Raymond F. Holden, Jr., M.D., and Kenneth G. Hink, B.S.E.E. (By Invitation), Wichita, Kan.

In comparing the effectiveness of devices used for the protection of workers against industrial and jet engine noise, promising results have been obtained with a modification of an old design (Plessner, 1885). This modified design, which comprises essentially an anodized aluminum sphere with an adjustable nylon retention knob, has been compared under laboratory conditions with two conventional types of insert ear protectors (bullet and V-51R designs) in 12 adults with normal hearing.

Subjects were tested individually in an efficient sound absorbent room measuring approximately 10 by 12 by 7 feet. A psychoacoustic method of testing was used; i.e., each subject's absolute threshold of hearing was determined before and after inserting ear protectors, the difference being the attenuation afforded by the device under test. Attenuations for each type of ear protector were measured in this manner for noise, octave band widths of noise, and tones. Speech reception thresholds in quiet and noise were measured, using recorded spondee words. The subjects received instructions and sound presentations through loudspeakers located within the sound absorbent room, and signaled awareness thresholds to observers in an adjoining control room.

It was apparent from the experimental data that the criteria employed in selecting the subjects (pure tone audiograms and otoscopic examination) did not insure uniformity of threshold responses, which displayed a definite tendency toward random distribution. By using analysis of variance techniques, it was possible to study the effects of the ear protectors independent of the effects due to subject differences. These studies indicated that the order of decreasing effectiveness for the ear protectors was: (1) ball-type, (2) bullet-type, and (3) V-51R type; octave by octave within the 20 to 9,600 c.p.s. range. The same was true for the speech range, 600 to 4,800 c.p.s. for white noise, and for pure tones. Attenuation of speech reception in quiet tended to be somewhat less than attenuation of white noise. Speech reception in noise appeared to be slightly improved and to about the same extent by all the devices tested.

It was concluded that the ball-type ear protectors were more uniformly effective than the other types which were examined.

61. A NEW ERYTHROPOIETIC SUBSTANCE DERIVED FROM LIVER

Daniel L. Horrigan, M.D., Cleveland, Ohio

Previous communications have described two patients with hypochromic anemia which was refractory to the usual hematopoietic agents but which responded to the oral administration of liquid extract of liver (Valentine). This anemia was accompanied in both patients by elevation in serum iron levels, which became normal during response to therapy The erythrocytes showed marked variation in size, shape, and color saturation The bone marrow showed erythroid hyperplasia with maturation arrest at an erythroblastic level The clinical characteristics of this anemia were very similar to those of a recently reported patient with anemia responsive to pyridoxine but not to oral crude liver extract

A previously unrecognized and as yet unidentified erythropoietic substance, which produces a hematologic response in one of these patients, has been isolated from the crude liver extract Procedures for the isolation of the active material include alcohol precipitation and ethyl acetate extraction of the crude extract followed by ion exchange and paper chromatographic techniques Activity in various fractions is demonstrated in the anemic subject by reticulocyte response, rapid erythrocyte regeneration, and decrease in serum iron concentration to normal values Hematologic relapse in about four months regularly follows cessation of therapy

From 90 ml of the crude extract (equivalent to approximately 680 Gm of unprocessed liver) about 1 0 mg of the active material is obtained this amount when given by mouth daily for 10 days produces an optimal hematologic response in the anemic subject A suboptimal response may follow daily oral doses of as little as 0 25 mg for 10 days

Although the nature of the active substance remains unknown, its ultraviolet spectral characteristics and microbiologic activity do not suggest an identity with any known erythropoietic agent, including pyridoxine and its derivatives Acid hydrolytic studies suggest a polypeptide structure within the molecule

The morphologic characteristics of the anemia in this patient suggest a metabolic defect in hemoglobin synthesis Also, the small amount of the active substance required to correct the anemia suggests that it is a cofactor The nature of this substance may further elucidate metabolic processes involved in hemoglobin synthesis

62 HYPOGAMMAGLOBULINEMIA AND CHRONIC LYMPHATIC LEUKEMIA

ROBERT P HUDSON, M D (BY INVITATION), AND SLOAN J WILSON, M D,
KANSAS CITY, KAN

Patients with chronic lymphatic leukemia (CLL) have decreased resistance to infection Rundles, Coonrad, and Arends (1954) found hypogammaglobulinemia in one of 15 patients with CLL Jim (1957) found hypogammaglobulinemia in 34 per cent of 50 CLL patients, but made no attempt to correlate the clinical course with this finding This study was undertaken to determine the incidence of hypogammaglobulinemia in patients with CLL and to correlate this decrease with (1) duration of disease, (2) type and amount of therapy, and (3) incidence and severity of infectious complications

Forty consecutive patients (Group A) with CLL were studied All had been followed at the University of Kansas since diagnosis Nineteen patients with chronic myelogenous leukemia and 7 with polycythemia vera (Group B) were also studied

Electrophoresis was by filter paper strip using Spinco apparatus and scanning with an Analytrol Normal gamma globulin by this method is 16 5 per cent with a range of 12 1 to 20 9 per cent Values less than 6 per cent were termed severe hypogammaglobulinemia

PAS, and was continued until the time the patient was discharged approximately 7 weeks later. The temperature remained at 100.5 to 101° F. (rectal) during the first week of Rimifon treatment, then fell to normal limits of 99.6° F. Thereafter the patient was afebrile.

Additional laboratory studies revealed that the patient's serum which was first tested with mycotic antigens on the fifteenth day of illness fixed complement to a dilution of 1:80, and had a collodion agglutination titer of 1:40 with antigens of *Histoplasma capsulatum.* As shown in column 1, Table I, complement-fixing antibody was demonstrable at this level with the soluble yeast phase antigen through the fourth week of hospitalization, after which the titer decreased steadily to 1:10 during the following 4 months. However, the collodion agglutination test was still positive in a serum taken approximately 6 months after onset. Cultures of a sputum specimen collected 21 days after onset yielded *H. capsulatum.* On the other hand, direct cultures as well as those made from the spleens and livers of Swiss albino mice injected with a sputum specimen obtained 4½ months after onset were negative for this agent. The patient's skin test was positive to Histoplasmin when this test was made during the sixth month of disease. A tuberculin test with second strength P.P.D. was also positive while that with the first was negative. In serial chest x-rays from the time of admission through the fourth month there was a gradual diminution of the nodular densities throughout both lung fields, as revealed by the film taken on Aug. 26, 1952 (Fig. 3).

The diagnosis of histoplasmosis was supported by clinical symptomatology, isolation of *H. capsulatum* from sputum, significant antibody levels to histoplasma antigens in both complement fixation and collodion agglutination tests, a positive skin test reaction to Histoplasmin and roentgenologic evidence of pulmonary infiltration.

CASE 2.—About one week after the onset of illness in Case 1, that patient's 32-year-old wife, although never hospitalized, became ill. In addition to generalized malaise, her initial symptoms were headache, diaphoresis, and anorexia. These were accompanied by tightness in the chest and cough productive of purulent sputum, with occasional hemoptysis. There was no nausea, vomiting, or diarrhea. Her recovery was uneventful and apparently complete within one week, although no specific therapy was prescribed.

TABLE II. SUMMARY OF SEROLOGIC DETERMINATIONS, CULTURE STUDIES, AND SKIN TESTS IN CASE 2 (WIFE)

TIME AFTER ONSET	COMPLEMENT FIXATION TITER			COLLODION AGGLUTINATION TITER (HISTOPLASMIN)	ORGANISM ISOLATED FROM SPUTUM	SKIN TEST WITH HISTOPLASMIN
	YEAST PHASE SOLUBLE ANTIGEN	WHOLE YEAST ANTIGEN	HISTOPLASMIN			
10 days	1:5	1:64	Negative	1:40		
15 days					H. capsulatum	
24 days	Negative	AC*	AC	>1:40		
43 days	Negative	--	--	1:40		
105 days	Negative	--	--	1:10	None	
191 days	Negative	1:32	Negative	1:10		Positive
4½ yr.	--	1:8	Negative	Negative		Positive†

*AC = Anticomplementary.

†76 by 76 mm. erythema; 26 by 26 mm. induration.

As shown in Column 1, Table II, a serum obtained 10 days after onset fixed complement to a titer of only 1:5 with histoplasma soluble yeast phase antigen. All others in the serial series on this patient were completely negative for complement-fixing antibody. On the other hand, the collodion agglutination test was strongly positive in the initial specimen as well as in those collected on the twenty-fourth and forty-third days. Following this, the reactions with this test gradually became less intense. Cultures of a sputum specimen obtained 15 days after onset were positive for *H. capsulatum.* However, and as in Case 1, the organism was not recovered either in direct culture or from the livers and spleens of mice inoculated with a sputum specimen collected 4 months after onset. The Histoplasmin skin test, made 6 months after onset, was positive. A single chest x-ray obtained during the fourth week failed to reveal any evidence of pulmonary infiltration.

which became normal during response to therapy. The erythrocytes showed marked variation in size, shape, and color saturation. The bone marrow showed erythroid hyperplasia with maturation arrest at an erythroblastic level. The clinical characteristics of this anemia were very similar to those of a recently reported patient with anemia responsive to pyridoxine but not to oral crude liver extract.

A previously unrecognized and as yet unidentified erythropoietic substance, which produces a hematologic response in one of these patients, has been isolated from the crude liver extract. Procedures for the isolation of the active material include alcohol precipitation and ethyl acetate extraction of the crude extract followed by ion exchange and paper chromatographic techniques. Activity in various fractions is demonstrated in the anemic subject by reticulocyte response, rapid erythrocyte regeneration, and decrease in serum iron concentration to normal values. Hematologic relapse in about four months regularly follows cessation of therapy.

From 90 ml. of the crude extract (equivalent to approximately 680 Gm. of unprocessed liver) about 1.0 mg. of the active material is obtained; this amount when given by mouth daily for 10 days produces an optimal hematologic response in the anemic subject. A suboptimal response may follow daily oral doses of as little as 0.25 mg. for 10 days.

Although the nature of the active substance remains unknown, its ultraviolet spectral characteristics and microbiologic activity do not suggest an identity with any known erythropoietic agent, including pyridoxine and its derivatives. Acid hydrolytic studies suggest a polypeptide structure within the molecule.

The morphologic characteristics of the anemia in this patient suggest a metabolic defect in hemoglobin synthesis. Also, the small amount of the active substance required to correct the anemia suggests that it is a cofactor. The nature of this substance may further elucidate metabolic processes involved in hemoglobin synthesis.

62. HYPOGAMMAGLOBULINEMIA AND CHRONIC LYMPHATIC LEUKEMIA

Robert P. Hudson, M.D. (By Invitation), and Sloan J. Wilson, M.D.,
Kansas City, Kan.

Patients with chronic lymphatic leukemia (CLL) have decreased resistance to infection. Rundles, Coonrad, and Arends (1954) found hypogammaglobulinemia in one of 15 patients with CLL. Jim (1957) found hypogammaglobulinemia in 34 per cent of 50 CLL patients, but made no attempt to correlate the clinical course with this finding. This study was undertaken to determine the incidence of hypogammaglobulinemia in patients with CLL and to correlate this decrease with (1) duration of disease, (2) type and amount of therapy, and (3) incidence and severity of infectious complications.

Forty consecutive patients (Group A) with CLL were studied. All had been followed at the University of Kansas since diagnosis. Nineteen patients with chronic myelogenous leukemia and 7 with polycythemia vera (Group B) were also studied.

Electrophoresis was by filter paper strip using Spinco apparatus and scanning with an Analytrol. Normal gamma globulin by this method is 16.5 per cent with a range of 12.1 to 20.9 per cent. Values less than 6 per cent were termed severe hypogammaglobulinemia.

Twenty-eight or 70 per cent of the CLL patients had hypogammaglobulinemia, the average having 8.2 per cent gamma globulin. Seven fell in the severe range and none had elevated gamma globulin. Of Group B, 3 (11.5 per cent) exhibited hypogammaglobulinemia and 3 had elevated gamma globulin.

The average duration of disease from diagnosis to electrophoretic study in the 28 CLL patients with hypogammaglobulinemia was 40.4 months compared to 21.8 months in the 12 patients with normal gamma globulin levels. Repeat determinations at 4-month intervals in 12 cases were lower than the initial value in every instance.

Treatment included steroids, urethane, TEM, P^{32}, chlorambucil, and x-radiation. In general, the more lympholytic therapy received, the lower the gamma globulin levels obtained. Since amount of treatment was determined largely by disease duration, no valid conclusions could be reached. Three patients with hypogammaglobulinemia had received no treatment.

Infectious complications in CLL requiring specific treatment occurred more frequently in patients with hypogammaglobulinemia.

It is concluded that patients with chronic lymphatic leukemia have an increased incidence of hypogammaglobulinemia which correlates best with duration of disease. No conclusion could be reached as to its relationship to therapy.

Erratum

In the article by Drs. IRWIN J. FOX and EARL H. WOOD entitled "Use of Methylene Blue as an Indicator for Arterial Dilution Curves in the Study of Heart Disease," which appeared in the October issue, pages 598-612, the fifth and sixth lines in the second paragraph on page 598 should be transposed.

The sentence should read as follows: "Evans blue dye (T-1824), the indicator commonly used, has the disadvantage of causing bluish discoloration of the skin and mucous membranes when given in doses exceeding approximately 0.5 mg. per kilogram[10] which, in addition to the cosmetic effect limiting the number of curves which may be performed, interferes with determination of blood oxygen saturation by oximetry and with the clinical evaluation of cyanosis, as, for example, after cardiac operation."

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CLINICAL AND EXPERIMENTAL

A FAMILY OUTBREAK OF HISTOPLASMOSIS

I Clinical, Laboratory, and Follow up Studies

Karl P Kolb, M D,* and Charlotte C Campbell, B S
Washington, D C

THIS report is concerned with an outbreak of histoplasmosis in a family of 4 persons living in Montgomery County, Maryland, within 5 miles of the city limits of Washington, D C As far as can be determined it is the first outbreak to have been detected in that area, although sporadic cases have been seen in the District of Columbia hospitals since 1939 [1]

Presented herein are the case histories and laboratory findings during the primary phase of illness and follow up x ray, serologic, and skin test studies on 3 of the patients, made 4½ to 5 years after clinical recovery The epidemiologic studies which led to the isolation of *Histoplasma capsulatum* from chicken compost to which the patients were exposed are described in a separate report [2]

CASE REPORT

Case 1 —A 33 year old white man was admitted to the hospital May 3, 1952, because of fever of 10 days' duration History revealed that his illness began with nausea, vomiting, diarrhea, headache, and generalized malaise Three days after onset the gastrointestinal symptoms subsided but the headache and malaise persisted He then noted chills and fever, accompanied by pain over the right lateral chest which was aggravated by deep breathing Aureomycin for a period of 2 days was prescribed by his private physician but failed to relieve his symptoms When Terramycin also failed, hospitalization was recommended

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Physical examination disclosed a well-developed, well-nourished white man who appeared acutely ill. His skin was flushed and his temperature was 103° F. His pulse rate was 92 per minute. He had a blood pressure of 140 systolic and 90 diastolic. There was an exanthem over his forehead. His pharynx was injected slightly and a few scattered râles were heard throughout both lung fields. There was no cardiomegaly but a Grade 1 apical systolic murmur was heard with the pulmonic second sound being greater than the aortic second. A small tender lymph node, 0.5 by 0.5 cm. was felt in his left axilla but there was no generalized lymphadenopathy. Examination of the abdomen revealed no masses. The spleen and liver were not enlarged.

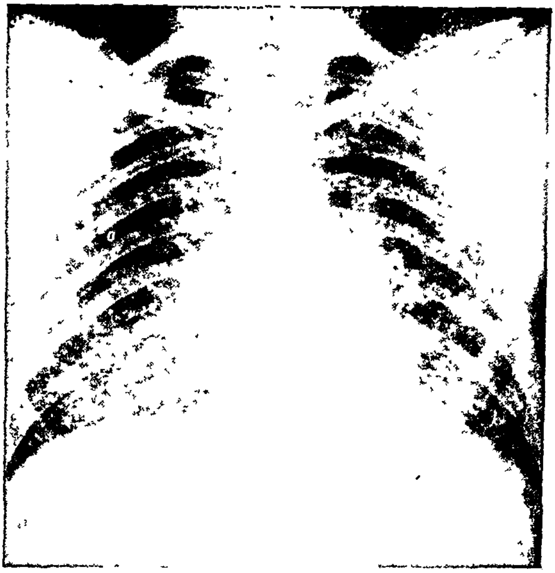

Fig. 1.—(Case 1.) Chest x-ray taken 12 days after onset of illness (May 4, 1952) reveals nodular and soft infiltrations throughout both lung fields in a miliary fashion.

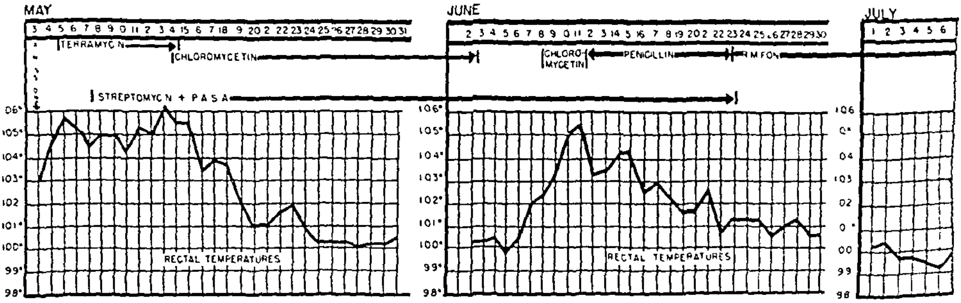

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Fig. 2.—(Case 1.) Composite therapy and temperature (maximum for the day) course.

At the time of admission the hemogram revealed a white blood count of 5,900 with a differential of 50 neutrophils, 8 stabs, 45 lymphocytes, 3 monocytes, and 2 eosinophils. The corrected sedimentation rate was 32 mm. per hour, the hemoglobin 14 Gm., and the hematocrit 41 per cent. Urinalysis was negative. Agglutinations for the usual bacterial infections and complement fixation tests for syphilis, the psittacosis-lymphogranuloma venereum group of viruses, and Q fever were also negative. Bromsulphalein, thymol turbidity, and van den Bergh tests all were within normal limits; however, the cephalin flocculation test was 4 plus. Cultures of sputum and aspirated bone marrow were negative for acid fast bacilli A chest x ray taken the day after admission revealed nodular and soft infiltrations of a circumscribed nature that were diffusely scattered throughout both lung fields in a miliary fashion (Fig 1)

The patient was isolated and treated supportively until specimens for further laboratory tests were obtained Treatment with Terramycin, as indicated in the composite picture of the therapy schedule and temperature course shown in Fig 2, was started on the second hospital day On the fourth hospital day streptomycin and PAS were also instituted because of the miliary appearance of the lung lesions However, when the patient s temperature remained elevated at 103 to 105° F through the tenth hospital day, Terramycin was discontinued and Chloromycetin started Following the second day of therapy with the latter drug the temperature steadily declined to approximately 100° F and continued at this level for the next 9 days At that time, Chloromycetin was discontinued Penicillin was then given for a period of 12 days, with a gradual lysis of temperature to 101 to 102° F (rectal) On the fifty first day of *hospitalization the hydrazine derivative of isonicotinic acid*, Rimifon (Hoffmann La Roche, Inc), in doses of 50 mg three times daily, was also substituted for streptomycin and

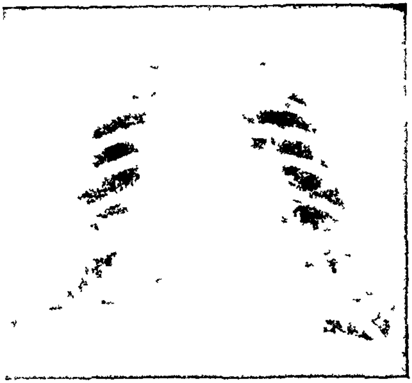

Fig 3 —(Case 1) Chest x ray taken four months after onset of illness (Aug 26 1952) shows nodular densities ranging from the minute up to 3 mm in diameter throughout both lung fields There is no evidence of consolidation cavitation or pleural effusion

TABLE I SUMMARY OF SEROLOGIC DETERMINATIONS, CULTURE STUDIES, AND SKIN TESTS IN CASE I (FATHER)

	COMPLEMENT FIXATION TITER					
TIME AFTER ONSET	YEAST PHASE SOLUBLE ANTIGEN	WHOLE YEAST ANTIGEN	HISTO PLASMIN	COLLODION AGGLUTINATION TITER (HISTO PLASMIN)	ORGANISM ISOLATED FROM SPUTUM	SKIN TEST WITH HISTO PLASMIN
15 days	1 80	-	-	>1 40		
21 days					H capsulatum	
28 days	1 80	-	-	1 40		
41 days	1 80	1 64	Negative	1 20		
71 days	1 40	1 32	Negative	1 40		
84 days	1 20	-	-	1 40		
111 days	1 20	1 8	Negative	1 40		
142 days	1 20	-	-	1 20	None	Positive
197 days	1 10	-	-	1 40		
4½ yr	-	1 16	Negative	Negative		Positive*

*76 by 35 mm erythema 20 by 20 mm induration

PAS, and was continued until the time the patient was discharged approximately 7 weeks later. The temperature remained at 100.5 to 101° F. (rectal) during the first week of Rimifon treatment, then fell to normal limits of 99.6° F. Thereafter the patient was afebrile.

Additional laboratory studies revealed that the patient's serum which was first tested with mycotic antigens on the fifteenth day of illness fixed complement to a dilution of 1:80, and had a collodion agglutination titer of 1:40 with antigens of *Histoplasma capsulatum*. As shown in column 1, Table I, complement-fixing antibody was demonstrable at this level with the soluble yeast phase antigen through the fourth week of hospitalization, after which the titer decreased steadily to 1:10 during the following 4 months. However, the collodion agglutination test was still positive in a serum taken approximately 6 months after onset. Cultures of a sputum specimen collected 21 days after onset yielded *H. capsulatum*. On the other hand, direct cultures as well as those made from the spleens and livers of Swiss albino mice injected with a sputum specimen obtained 4½ months after onset were negative for this agent. The patient's skin test was positive to Histoplasmin when this test was made during the sixth month of disease. A tuberculin test with second strength P.P.D. was also positive while that with the first was negative. In serial chest x-rays from the time of admission through the fourth month there was a gradual diminution of the nodular densities throughout both lung fields, as revealed by the film taken on Aug. 26, 1952 (Fig. 3).

The diagnosis of histoplasmosis was supported by clinical symptomatology, isolation of *H. capsulatum* from sputum, significant antibody levels to histoplasma antigens in both complement fixation and collodion agglutination tests, a positive skin test reaction to Histoplasmin and roentgenologic evidence of pulmonary infiltration.

CASE 2.—About one week after the onset of illness in Case 1, that patient's 32-year-old wife, although never hospitalized, became ill. In addition to generalized malaise, her initial symptoms were headache, diaphoresis, and anorexia. These were accompanied by tightness in the chest and cough productive of purulent sputum, with occasional hemoptysis. There was no nausea, vomiting, or diarrhea. Her recovery was uneventful and apparently complete within one week, although no specific therapy was prescribed.

TABLE II. SUMMARY OF SEROLOGIC DETERMINATIONS, CULTURE STUDIES, AND SKIN TESTS IN CASE 2 (WIFE)

TIME AFTER ONSET	COMPLEMENT FIXATION TITER			COLLODION AGGLUTINATION TITER (HISTOPLASMIN)	ORGANISM ISOLATED FROM SPUTUM	SKIN TEST WITH HISTOPLASMIN
	YEAST PHASE SOLUBLE ANTIGEN	WHOLE YEAST ANTIGEN	HISTOPLASMIN			
10 days	1:5	1:64	Negative	1:40		
15 days					H. capsulatum	
24 days	Negative	AC*	AC	>1:40		
43 days	Negative	--	--	1:40		
105 days	Negative	--	--	1:10	None	
191 days	Negative	1:32	Negative	1:10		Positive
4½ yr.	--	1:8	Negative	Negative		Positive†

*AC = Anticomplementary.

†76 by 76 mm. erythema; 26 by 26 mm. induration.

As shown in Column 1, Table II, a serum obtained 10 days after onset fixed complement to a titer of only 1:5 with histoplasma soluble yeast phase antigen. All others in the serial series on this patient were completely negative for complement-fixing antibody. On the other hand, the collodion agglutination test was strongly positive in the initial specimen as well as in those collected on the twenty-fourth and forty-third days. Following this, the reactions with this test gradually became less intense. Cultures of a sputum specimen obtained 15 days after onset were positive for *H. capsulatum*. However, and as in Case 1, the organism was not recovered either in direct culture or from the livers and spleens of mice inoculated with a sputum specimen collected 4 months after onset. The Histoplasmin skin test, made 6 months after onset, was positive. A single chest x-ray obtained during the fourth week failed to reveal any evidence of pulmonary infiltration.

The diagnosis of histoplasmosis in this case was based on the isolation of *H capsulatum* from sputum obtained early in the course of illness, the significant antibody levels demonstrated by the collodion agglutination test, and a positive skin test reaction to Histoplasmin

CASE 3—Also about one week after the onset of illness in Case 1, the patient's 14 year old son became ill Again, the symptoms were acute, with pain in the left chest, cough with production of purulent bloodstreaked sputum, anorexia, generalized malaise, and headache as the predominant ones Temperature elevations of 103° F were recorded during the first week However, the patient made a rapid recovery and was able to return to school after an absence of approximately two weeks Therapy included a total dosage of 2 5 Gm Terramycin which was given every 4 hours in 250 mg doses

TABLE III SUMMARY OF SEROLOGIC DETERMINATIONS, CULTURE STUDIES, AND SKIN TESTS IN CASE 3 (SON)

TIME AFTER ONSET	COMPLEMENT FIXATION TITER YEAST PHASE SOLUBLE ANTIGEN	WHOLE YEAST ANTIGEN	HISTOPLASMIN	COLLODION AGGLUTINATION TITER (HISTOPLASMIN)	ORGANISM ISOLATED FROM SPUTUM	SKIN TEST WITH HISTOPLASMIN
10 days	1 40	1 64	Negative	1 20		
15 days					None	
24 days	1 40	1 256	1 32	1 40		
43 days	1 20	AC*	AC	1 10		
105 days	1 10	AC	AC	1 10		
191 days	Negative	1 128	1 256+	1 10		Positive
4½ yr	--	1 16	1 8	Negative		Positive†

*AC = Anticomplementary
†50 by 50 mm erythema 40 by 50 mm induration

As noted in Column 1, Table III, the complement fixation titer with histoplasma soluble yeast phase antigen was 1 40 in specimens taken on the tenth and twenty fourth days after onset This level decreased to 1 20 and 1 10 in serums collected at approximately 6 and 15 weeks, respectively In a serum specimen taken 6 months after onset no complement fixing antibody was demonstrable Collodion agglutination tests in the tenth and twenty fourth day serum specimens were also positive, whereas results with later specimens were equivocal. *H capsulatum* was not isolated from the single sputum collected about 15 days after onset of symptoms In the single chest x ray obtained during the fourth week there was no evidence of pulmonary infiltration or hilar adenopathy The patient, however, did have a positive reaction to a skin test with Histoplasmin when this was made approximately 6 months after onset of illness

Although the diagnosis of histoplasmosis in this case was not supported by the recovery of *H capsulatum*, the positive serologic and dermal sensitivity tests strongly indicated that the boy's infection was also due to this fungus, particularly since symptomatologically and in time of onset it closely simulated culturally verified Cases 1 and 2

TABLE IV SUMMARY OF SEROLOGIC DETERMINATIONS, CULTURE STUDIES, AND SKIN TESTS IN CASE 4 (SISTER IN LAW)

TIME AFTER ONSET	COMPLEMENT FIXATION TITER (YEAST PHASE SOLUBLE ANTIGEN)	COLLODION AGGLUTINATION TITER (HISTOPLASMIN)	ORGANISM ISOLATED FROM SPUTUM	SKIN TEST WITH HISTOPLASMIN
13 days	1 10	1 10		
18 days			None	
27 days	1 80	>1 40		
46 days	1 80	1 40		
108 days	1 20	1 20	None	
194 days	Negative	1 5		Positive
4½ yr	--	--		--

CASE 4.—The fourth person involved in this series was the 27-year-old sister-in-law of Case 1 who resided with the family, and who was actively ill for only 2 or 3 days. Symptoms began about 2 days after the onset of illness in Case 1 and, although of shorter duration and less severe, they were similar to those experienced by the 3 other members of the family. She was away from her job for just one week. Since recovery was uneventful, no medication was prescribed.

In this patient, as illustrated in Column 1, Table IV, the antibody levels were very low in the earliest serum obtained. Fourteen days later, however, the complement fixation titer had increased to 1:80 and the collodion agglutination test had become markedly positive. These high antibody levels persisted for at least 3 more weeks and then, as in the other cases, gradually decreased. The patient was sensitive to Histoplasmin when tested 6 months after onset, in spite of the fact that cultures of two sputum specimens were negative for *H. capsulatum.* The single chest x-ray taken during the fourth week was negative.

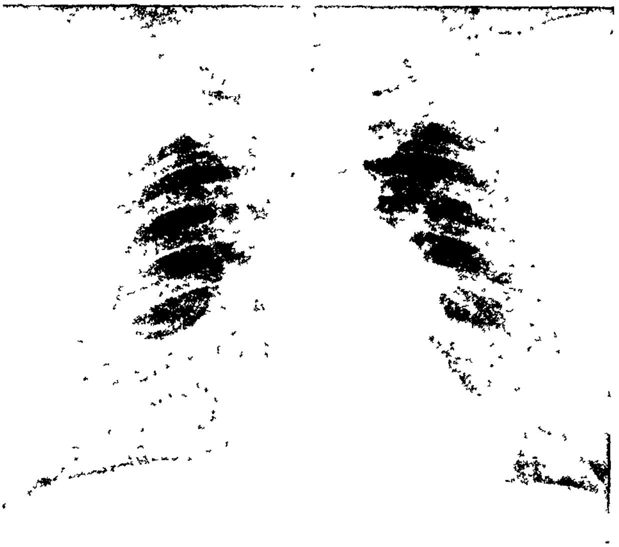

FIG. 4.—(Case 1.) Chest x-ray taken approximately 5 years after onset of illness (June 3, 1957) discloses no appreciable change from Fig. 3. The variable nodular densities are of comparable size, and no true calcification is seen.

The diagnosis of probable histoplasmosis in this case was based on a demonstrated increase in antibody levels to histoplasma antigens, a positive skin test to Histoplasmin, and on the similarity in the time of onset and clinical symptoms observed in Cases 1, 2, and 3.

The Follow-Up.—Additional data were obtained in 3 of the 4 cases 4½ to 5 years after the onset of their illnesses.

CASE 1.—At the time of the follow-up, the father appeared to be a vigorous, healthy man. His weight was 210 pounds, which represented a gain of 40 pounds over that maintained prior to his hospitalization with histoplasmosis. He resumed his occupation as butcher early in 1953 and has been employed continuously ever since. He has not had colds or further upper respiratory disturbances. As earlier noted, the serial chest x-rays (Fig. 1) from the time of his hospital admission in May, 1952, until 4 months thereafter showed a gradual and continuous diminution of the nodular densities throughout both lung fields (Fig. 3), but in the five years intervening there was no appreciable change. In a film taken in June, 1957, the nodular densities still varied from minute to 3 mm. in diameter and no true calcification was seen (Fig. 4). As shown in Column 2, Table I, this man maintained a C.F. antibody titer of 1:16 with whole cell histoplasma yeast phase antigen.

His skin test reaction to Histoplasmin also remained markedly positive Complement fixation and collodion agglutination tests with the mycelial filtrate antigen, Histoplasmin, were negative

Case 2—His wife also appeared to be well and healthy, and for the past 3 years has been employed as a stenographer in a large metropolitan bank Approximately one year after the primary phase of her histoplasma infection she began to have low back pain In the pyelograms made at that time there was a persistent filling defect in the region of the pelvis on the right due to some organic lesion Whether or not this was an extrapulmonary focus of *H capsulatum* was not determined The symptoms eventually disappeared and there has been no recurrence A chest x ray taken in December, 1956, was negative, as was the earlier film made during the fourth week after onset of infection with *H capsulatum* in 1952 Evidence of this patient s infection persists only in the residual C F antibody titer of 1 8 with the whole cell yeast phase antigen (Column 2, Table II) and her strongly positive skin test reaction to Histoplasmin (Table II)

Case 3—The son currently is an enlisted man in a branch of the United States Armed Forces In the follow up chest x ray in December, 1956, there was a disseminated distribution of tiny calcified nodules, creating a "snow storm" pattern throughout both lung fields, regarded as very characteristic of healed miliary histoplasmosis (Fig 5) This was in contrast to the negative findings during the fourth week of infection A serum specimen also obtained in December, 1956, fixed complement to titers of 1 16 and 1 8 with the whole cell yeast phase antigen and Histoplasmin, respectively His skin test reaction to Histoplasmin was strongly positive (Table III) A simultaneous skin test with P P D was negative

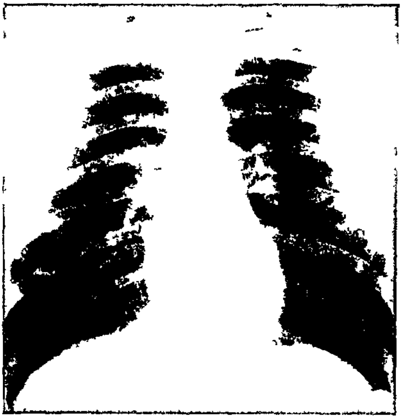

Fig 5—(Case 3) Chest x ray taken 4½ years after onset of illness (Dec 22 1956) shows a disseminated distribution of tiny calcified nodules throughout both lung fields

Case 4—The sister in law apparently is also healthy and well However, this patient moved to a distant state about two years after her histoplasmosis infection, and we were unable to gain her cooperation for follow up studies

METHODS

Serologic Tests—The complement fixation (C F) and collodion agglutination (C.A.) tests employed were carried out as described in earlier reports[3 4] The antigen used for the C.F. test in the primary phase of the study was the physiologic saline soluble component of yeast phase cells of *H. capsulatum* following disruption by grinding.[4] The whole cell yeast phase antigen and Histoplasmin,* a filtrate of the filamentous growth of the organism, were employed in follow-up C.F. tests.[5] Histoplasmin was the only antigen used in the C.A. test.

Skin Tests.—Follow-up skin tests were made with a 1:100 dilution of Histoplasmin.†

Culture Studies.—Early morning sputum samples were emulsified in 5 to 10 ml. physiologic saline solution containing 1,000 and 2,000 units of penicillin and streptomycin per milliliter, respectively. After an incubation period of one hour at 37° C., 0.5 ml. quantities were inoculated intraperitoneally into each of five 18 to 20 gram male albino mice and planted on brain heart infusion agar (Difco) containing 20 and 40 units of penicillin and streptomycin per milliliter, respectively, and one per cent dextrose. Plates of inoculated medium were incubated at room temperature for a period of 4 weeks. The mice were observed for 4 weeks, at which time all survivors were sacrificed and their livers and spleens cultured for *H. capsulatum.* The spleens and livers of mice dying after the seventh day were also cultured. Colonies appearing after the tenth day were transferred to Sabouraud's dextrose agar (Difco) and incubated at room temperature for final identification.

DISCUSSION

The clinical courses of the 4 persons involved in this family outbreak of primary, pulmonary histoplasmosis, the first to be reported from the District of Columbia area, were remarkably similar to those described in the epidemics report by Loosli and associates[6] and Furcolow and Grayston.[7] In 3 of the 4 persons, the symptomatology was relatively mild, transitory, and clinically indistinguishable from that in mild upper respiratory infections of viral or bacterial etiology. In fact, had it not been for the grave illness in Case 1 it is doubtful that the milder illnesses would have been ascribed to any specific agent, much less to the fungus, *H. capsulatum.* Despite the variation in the degree of severity, however, all made spontaneous and apparently complete recoveries.

In order to establish that an outbreak of pneumonitic illnesses is due to *H. capsulatum,* it is sometimes necessary to rely on indirect criteria as outlined in the studies of epidemics by Grayston and Furcolow.[8] In the present one, however, the rapid demonstration of agglutinins and complement-fixing antibodies with histoplasma antigens in early sera from each of the patients led to immediate cultural studies. The latter yielded *H. capsulatum* not only from two of the persons involved, but ultimately also from the contaminated chicken compost to which all 4 had been recently exposed.[2] Skin tests with Histoplasmin were also positive.

This series of cases also provided additional evidence that therapy with antibacterial antibiotics has little if any beneficial effect on the course of primary, pulmonary histoplasmosis. Two of the four cases were untreated and a third received a total dosage of 2.5 Gm. Terramycin. The fourth case was treated with a variety of drugs, including Terramycin, Chloromycetin, penicillin, Rimifon, streptomycin, and PAS. In spite of this, this patient's stormy, prolonged course was essentially similar to that in other reported cases of equal severity who recovered without benefit of specific therapy. Loosli[9] and Silverman and his associates[10] describe severe infections in which recovery occurred without the use of antibacterial antibiotics or drugs of any kind. Moreover, there is some evidence that streptomycin, at least, actually enhances rather than inhibits the growth of mycotic agents[11]

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*Lot H-15: Parke, Davis & Company, Detroit, Mich.

†Lot 029386-F-029975-F: Parke, Davis & Company, Detroit, Mich.

Of special interest were the follow up findings relating to pulmonary involvement Extensive infiltration (Fig 1) was observed during the initial phase of the study only in the person with the most severe illness Although there was considerable clearing during the first 4 months (Fig 3), since that time there has been no essential change either in the areas of nodular densities or size of the individual lesions Nor is there evidence of calcification (Fig 4) In contrast, the follow up film in Case 3 revealed a typical "snow storm" pattern of calcification (Fig 5) although the single film obtained on this patient during the fourth week after onset was negative The initial and follow up films in Case 2 were both clear Whether infiltrates might have been present at some time prior to or later than the fourth week in either of these two milder cases, of course, cannot be ascertained From the still negative skin test reaction to P P D and the extensive calcification in Case 3, however, it is not unlikely that at one time there was considerable infiltration in this patient For this reason, serial chest x rays appear to be as important as serial serologic and repeated cultural studies in following the early suspected and verified cases of histoplasmosis The reported absence of pulmonary infiltration during the primary episode even in relatively mild infections perhaps should be viewed with caution unless there are serial x rays at rather close intervals to substantiate this point

Serologic tests, as noted above, were used to good advantage in the detection and study of these cases Cultural studies were initiated only after it was known that serologic tests were positive The importance of obtaining specimens (sputum, gastrics and blood) for culture during an acute phase of this infection has perhaps received too little emphasis Experience in this laboratory reveals that it is during the early stage of infection that the organism is most readily isolated In this regard the collodion agglutination test is especially useful This test which can be performed in any hospital laboratory within a period of 3 hours directs attention to the possibility of primary pulmonary histoplasmosis during a phase of active infection when cultural and additional serologic studies yield *the most definitive and valuable information Agglutinins, moreover, are* usually demonstrable by the time symptoms begin to occur even in cases which are not sufficiently severe for the development of detectable complement fixing antibodies[12, 13]

As illustrated in Tables I, II, and III the results of complement fixation tests vary with the type of antigen, duration and type of disease, and other as yet undetermined factors For example, the reactions with whole yeast phase antigen in sera from Case 1 were essentially the same as those with the soluble antigen, whereas those in sera from Cases 2 and 3 were considerably higher with the whole cell preparation Later sera from Case 3 also reacted to a titer exceeding 1 256 with Histoplasmin, in contrast to negative findings with this antigen in Cases 1 and 2 The importance of employing different types of antigenic preparations is discussed in an earlier report[9] There is at the present time little doubt that the whole cell antigen is superior to the soluble yeast phase component, that the reactions obtained with the whole yeast cell antigen also sometimes differ markedly from those obtained with Histoplasmin, and that until these differences are resolved both types of antigens should be employed in complement fixation tests for histoplasmosis. However, it is not unusual for a low residual C.F. antibody titer or a marked skin test sensitivity to persist for an, as yet, undetermined number of years following a primary infection, as illustrated by the findings in these cases. Their interpretation awaits the close study and follow-up of an increasing number of cases from the time of onset of infection as exemplified by the investigations of Loosli and his associates.[6]

SUMMARY

An epidemic of primary, pulmonary histoplasmosis in a family of 4 persons living near Washington, D. C., is described. The father was critically ill for a period of 3 months, whereas the infections in his wife, son, and sister-in-law were only mild to moderately severe. All recovered. *Histoplasma capsulatum* was isolated from the sputums of the husband and wife. Elevated titers of agglutinins and C.F. antibodies were demonstrated in the sera of all 4 persons and all were skin test positive to Histoplasmin. Although the father was treated with a variety of antibiotic agents, none of the agents appeared to alter his stormy course. Follow-up chest x-rays and skin and serologic tests were obtained 4½ to 5 years later on 3 of the patients. In the son's film there was a typical "snow storm" pattern of calcification (Fig. 5) whereas in the father's there was no calcification and no appreciable change in nodular densities compared with 5 years before (Figs. 3 and 4). The film on the wife was negative. Residual C.F. titers of 1:8 and 1:16 and strongly positive skin test reactions to Histoplasmin persisted in all 3 persons. Studies leading to the isolation of *H. capsulatum* from contaminated chicken compost to which 4 persons were exposed approximately two weeks prior to onset of their infections are described in a separate report.[2]

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A FAMILY OUTBREAK OF HISTOPLASMOSIS

II. Epidemiologic Studies

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THIS report is concerned with the epidemiologic aspects of an outbreak of histoplasmosis involving all the members of a family.[1] These individuals were residents of Glenmont, a suburban housing development situated within 5 miles of the city limits of Washington, D. C., in Montgomery County, Maryland. The epidemic, although similar in many respects to others earlier reported,[2-4] was of special interest because it occurred in a geographic area of "low" endemicity and involved persons who lived in a suburban rather than rural area. These findings are conspicuous only in that they invalidate certain not altogether warranted impressions that have arisen during the last 10 years concerning the acquirement of this infection.

The purpose of this report, therefore, is not only to describe epidemiologic studies in an additional outbreak of histoplasmosis, but also to emphasize that (1) the geographic distribution of *Histoplasma capsulatum* has not been fully outlined, (2) maps of endemicity in many instances reflect the extent to which the disease has been investigated in particular localities rather than actual prevalence of the infection, thus, in areas of purportedly low endemicity the real prevalence of the infection is often unknown, (3) exposure to the etiologic agent in nature is not necessarily associated with dusty environments or uncommon activities of prolonged duration, and (4) infections are not limited to rural residents or other isolated groups.

The Family.—This family comprised 4 persons: the husband, his wife, their 14-year-old son, and a sister-in-law. The father was employed as a butcher in an Army commissary and the wife and sister-in-law as stenographer and clerk typist, respectively, by different commercial firms. The son attended high school. Week ends were used for gardening, housekeeping, marketing, and similar duties. The family occasionally took automobile rides on Sunday, but on these occasions did not visit farms or take walks over country terrain. There had been no vacations, either separately or together, for at least 8 months. The father, at the time of onset of illness, was terminating a convalescent period following surgery for hernia but other members of the family had been healthy and well prior to onset of their respiratory infections.

The Outbreak.—In April, 1952, each of these persons experienced an episode of primary pulmonary histoplasmosis.[1] The diagnosis was unequivocally established in the husband and wife by the isolation of *H. capsulatum* from their sputums and presumptively in the son and sister-in-law by a demonstrated increase in histoplasmin antibody as well as similarity in symptomatology and time of onset to the two verified cases. The husband's episode was one of extreme gravity and required a 3 month period of hospitalization for recovery. The son's, wife's, and sister-in-law's illnesses, requiring only 1 to 2 weeks of bed rest at home, were considerably less severe. An investigation to find the point source in nature from which these infections were acquired was initiated at once. All available evidence indicated that this source existed in the District of Columbia area.

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The Environment.—The Glenmont housing development consisted of an area approximately two miles square. Modest clapboard houses of relatively contemporary design occupied lots approximately 60 feet wide and 100 feet deep, and were fronted by concrete sidewalks and paved streets. At the time of the outbreak, there was no evidence of construction involving the use of bulldozers or other dust-raising equipment within a radius of one-half mile. The narrow front lawns were sodded and well kept.

The house in which this family had lived for the past 5 years was essentially the same as many others in the immediate and surrounding blocks. Like these, it was situated near the front of the lot to allow space for small vegetable or flower gardens as well as additional lawn in the rear. Because of the season many of the gardens had recently been plowed and planted. This family's garden adjoined that of the neighbor's to the rear and those of the neighbors' on both sides. An occasional lot was partitioned by a low wire fence. More often, there was no demarcation, and the individual properties were continuous, permitting free access from one to the other. The few trees appeared to be young and vigorous.

FIELD STUDIES

Early discussion with the father revealed that while he could recall no particular activity in which the family had participated collectively, he and his son had cleaned the basement of their home approximately 10 days before the onset of their illnesses. This had proved to be an especially dusty and time-consuming task as the son, who was an amateur naturalist, used it as a workshop. There he prepared and preserved for display or kept as pets for indefinite periods birds, their nests and eggs, and small rodents which he collected during his frequent trips to a little wood about one mile south of the development. Neither the wife nor the sister-in-law was at home when the dust was at its highest concentration, but both made brief excursions to the basement later in the day. Moreover, sufficient dust had escaped to the upper story to necessitate a thorough cleaning of the house. It was also recalled that 3 baby squirrels were brought into the house about one month before the onset of these illnesses. The animals died after several days, allegedly of malnutrition, but actually of unknown causes. Other possible contributors to the outbreak were the several birds' nests, wings from a sparrow hawk, the large eggshell collection on display in the boy's room, and the dog and three cats owned by the family. The technique for emptying eggshells was especially suspect since it involved bathroom towels in common use. Finally, it was learned that game birds, following removal of their wings for preservation, were often eaten by all persons concerned in the outbreak.

In spite of this array of possibilities, attention was focused on the basement as the most likely source. This was a large, noncompartmented room with a concrete floor and walls of the cinder block type. It was accessible only by a stairway leading from the kitchen. Relatively dry and well lighted, it contained the usual permanently installed laundry tubs and furnace, but no storage bins or chests. Because of the recent cleaning, little remained to be collected as specimens for mycologic study. Owl pellets that were overgrown with a species of nonpigmented fungus were found on the floor in one corner. These, a 50 by 70 cm. mat of fungus about two feet distant from the pellets, and a green mold covering one of the glass eyes on a stuffed buffalo head were the only specimens returned to the laboratory for study.

A second trip to the home was made three days later. On this occasion specimens from the surrounding environs as well as additional ones from the basement were collected. These included (1) washings sponged from each of the basement walls, (2) soil samples from 4 different areas in the backyard and garden, 3 were selected because they were the possible but completely forgotten burial sites for the baby squirrels and the fourth because it comprised the floor of a wire chicken pen used for caging captured birds and only rarely for housing chickens prior to slaughter, (3) nesting materials from the wooden box in which the baby squirrels died, (4) a walnut shell filled with mycelium also found in this box, (5) a pigeon's nest from a high rafter in a deserted barn approximately one mile distant from the house, from which the son often collected specimens of eggs and birds, and (6) the owl's nest, by this time abandoned, from which the pellets found earlier on the basement floor originated.

As shown in Table I, *H. capsulatum* was not recovered from any of these specimens. Negative results were also obtained by two other investigators to whom portions of many of these materials were sent.* It was of interest, however, that *Cryptococcus neoformans* was isolated from the pigeon's nest by each of the persons who studied it.[5, 6]

TABLE I. SUMMARY OF ISOLATION STUDIES FOR HISTOPLASMA CAPSULATUM ON SPECIMENS FROM SUSPECTED EXPOSURE SOURCES (GLENMONT OUTBREAK, MONTGOMERY COUNTY, MD.)

NO.	SPECIMENS	MYCOTIC PATHOGEN ISOLATED CULTURE	 MOUSE INOCULATION
	Collected May, 1952		
1	Owl pellets	None	None
2	Mycelial mat (50 × 70 cm.)	None	None
3	Green mold on glass eye of buffalo head	None	None
4	Basement wall washings	None	None
5	Soil sample No. 1	None	None
6	Soil sample No. 2	None	None
7	Soil sample No. 3	None	None
8	Soil sample No. 4 from chicken coop	None	None
9	Nesting material in squirrel box	None	None
10	Mycelium filled nutshell	None	None
11	Owl's nest	None	None
12	Pigeon's nest	*C. neoformans*	*C. neoformans*
	Collected December, 1956		
13-21	Soil samples from garden	Not done	None
22	Soil sample from chicken house floor	Not done	*H. capsulatum*
23	Soil sample from chicken house floor	Not done	*H. capsulatum*
24	Soil sample from chicken house floor	Not done	*H. capsulatum*
25-27	Soil sample from chicken house floor	Not done	None
28-30	Soil with crumbling wood from slatted platform in chicken house	Not done	None

In spite of these negative findings, it was still believed that the basement was the only common source to which all of these persons could have been exposed. Our failure to recover *H. capsulatum* from any of the above specimens was attributed to the fact that the offending focus was removed from the basement during the cleaning operation before our first trip to the house. For this reason, further studies did not appear to be warranted.

In December, 1956, approximately 4½ years after the onset of these infections, follow-up x-rays and skin and serologic tests on 3 of the 4 persons were obtained.[1] When the father returned for his follow-up test, he was accompanied by his ailing mother-in-law in order that skin and serologic tests might also be made on her. When it was disclosed that the mother-in-law resided on a small farm near Waycross, Georgia, and that she tended a small flock of chickens, considerable emphasis was placed on whether or not she also cleaned the hen house. On being advised of the close association of *H. capsulatum* spores and soils enriched with chicken excreta,[7] the father instantly recalled that he had collected, hauled, and spread chicken manure on his own garden the day before cleaning the basement in his home.

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*I am indebted to Dr. C. W. Emmons, National Institutes of Health, Bethesda, Md., and to Dr. Libero Ajello, Communicable Disease Center, Chamblee, Ga., for their valued assistance in these studies.

Armed with this heretofore undisclosed information, a third trip was made to collect soil samples. Soils were taken from nine areas in the garden, which during the intervening years had been repeatedly plowed, fertilized, and planted. Since the chicken house from which the manure was initially obtained was not more than one mile east of this suburban housing development, specimens of soil were also collected from it. This small dilapidated structure, no longer suitable for housing chickens because of a leaky roof and missing planks on the sides, was empty except for a barrel and a few chunks of fire wood. Six samples of soil were collected from various areas of the moist dirt floor and 3 additional ones from a slightly elevated platform on which the crumbling fire wood was stacked.

As shown in Table I, *H. capsulatum* was readily recovered from mice inoculated with 3 of the 6 soil samples collected from the dirt floor of the chicken house. Specimens from the slatted platform as well as the garden areas were negative. It is of interest to note that the organism was isolated in one instance as early as two weeks after injection, indicating that even though 4½ years had elapsed the spores were still present in high concentration in this particular sample.

LABORATORY STUDIES

1. *Materials from Sources in Nature.*—

Soil samples: For those samples collected during the initial phase of this study three different preparation techniques were employed in order to utilize each of the various modifications described by Emmons,[8] Zeidberg and Ajello,[9] and Larsh and associates[10] for isolating *H. capsulatum* from soil. Only the method described by Larsh and associates[10] was used on the later samples. These suspensions were used only in mice.

Bird and animal nests: Approximately 2 grams of twigs, leaves, feathers, and soil were selected from different parts of the nests, broken into minute fragments, placed in a 100 ml. cylinder containing 30 ml. of physiologic saline, and shaken vigorously. After 10 minutes, the supernatant fluid was siphoned from the sedimented larger particles and centrifuged at 5,000 r.p.m. for 5 minutes. The sediment from the latter, resuspended in 10 ml. of saline, comprised the inoculating suspension for mice. No direct platings on culture medium were made.

Basement wall washings: Areas, approximately 4 feet square, on each of the 4 basement walls were swabbed with sterile sponges moistened and later rinsed in 1,000 ml. sterile, distilled water. Debris from the latter was collected on millipore filters (Type HA) and resuspended in 10 ml. saline.[11] Media as well as mice were inoculated with these suspensions.

Miscellaneous specimens: Other specimens such as owl pellets, the several specimens of fungi, nutshells, etc., were each suspended in 30 ml. of physiologic saline, shaken vigorously, and allowed to stand in 100 ml. cylinders for one hour. Aliquots of 5 ml. were removed separately from the surface, middle, and supernate-sediment interface of each suspension for injection into mice and inoculation of selective media.

2. *Media.*—Brain heart infusion agar (Difco), to which glucose, penicillin, and streptomycin were added in final concentrations of 2 per cent and 20 and 40 units per milliliter of medium, respectively, was the sole substrate for culturing livers and spleens of inoculated mice. Sabouraud's agar containing cycloheximide was used for the direct culture of many of the specimens collected from nature.[12]

3. *Animal Inoculations.*—Male albino mice in groups of 10 and weighing 18 to 20 grams were used. They were inoculated intraperitoneally with 0.5 or 1.0 ml. volume of the prepared specimens, depending upon the quantity available. All were treated daily for 7 days following inoculation with 1,000 units of penicillin and 2,000 units of streptomycin. The animals were observed for a period of 4 weeks, at which time half of the survivors were sacrificed and their livers and spleens cultured for *H. capsulatum*. The remaining mice were sacrificed and their livers and spleens cultured at the end of 8 weeks. The spleens and livers of mice which died after the seventh day were also cultured.

Except for those soil samples collected during the follow-up phase of the study, this series of procedures was repeated 3 times in this laboratory on separate aliquots of each of the specimens collected from nature. In one of these trials, 5 per cent hog gastric mucin was substituted for physiologic saline as the suspending menstruum for materials injected into mice.[13, 14]

DISCUSSION

Although forests, chickens, birds, rodents, and even domestic pets were involved in this epidemiologic investigation—and all are implicated as possible contributors to the distribution of *H. capsulatum* in nature[1, 15-20]—the agent finally was recovered from the source most commonly associated with primary, pulmonary infections of histoplasmosis, soils enriched with the excreta of chickens or other fowl.[5-7]

It was nevertheless of interest to learn that in the contracting of these infections the time of exposure was so brief and the source so usual that neither was recalled until specific questions were asked. In this instance, the initial period of exposure could not have exceeded a period of one hour and, unlike more dramatic undertakings such as speleologic expeditions and the cleaning of silos, water towers, or even dusty basements,[2-4] it was associated with a very commonplace task. The father, also accompanied by his son on this occasion, had driven to the nearby farm, hurriedly scooped the soil into small kegs, transported it home in the trunk of his car and deposited it on the garden. Yet the ready recovery of *H. capsulatum* from 3 of the 6 soil samples collected several years later from this original source leaves little doubt that it was in the execution of this incidental chore that the near fatal and moderately severe illnesses were incurred. For the wife and sister-in-law, the means of exposure was even less impressive. Neither was directly involved in this task. They merely pulled weeds in the garden within the next few days.

Nor can it be assumed that these were the only cases among the many persons having free access to this garden. The initial studies included a serum from a 12 year old boy who lived across the street and whose upper respiratory infection coincided in time of onset and duration with that of the son's. A titer of 1:128 in serologic tests left little doubt that his infection was one of histoplasmosis acquired in a manner similar to the others because of his frequent visits to this home. Therefore, in our excessive concern over the dusty basement we had failed to investigate the more serious illness in the neighbor who lived to the rear, despite the fact that her bout with bronchopneumonia was sufficiently severe to require 3 weeks of hospitalization. She, indeed, had not visited this family, but her garden directly adjoined their contaminated plot. In retrospect, it is thus not unlikely that her infection and possibly some of the milder ones that beset residents of this community was due to exposure to this at least temporary focus of *H. capsulatum.*

This series of cases comprises only a minor part of the evidence that the District of Columbia area is one of high endemicity for histoplasmosis, contrary to the relatively low prevalence assigned to it on distribution maps.[3] *Histoplasma capsulatum* is isolated repeatedly from the soils of nearby communities by Emmons as well as ourselves.[5, 21,23] Moreover, as disclosed by our own serologic studies carried out cooperatively with physicians in this area, there were 33 cases with serologic titers of 1:16 or above with histoplasma antigens in residents of the District of Columbia and adjoining states within the past year. Patients in military installations in these states account for 128 additional cases with a presumptive serologic diagnosis of histoplasmosis within the same period.[23] These figures included only those persons with illnesses of sufficient severity to require hospitalization.

In view of the above, it is therefore pertinent to point out that distribution maps might not be entirely accurate for other regions assigned a low prevalence, but where, as in the District of Columbia, the incidence is actually unknown. In this regard, it is not generally recognized that skin test surveys with Histoplasmin, the principal method for determining the distribution of this agent, have not been carried out with the same intensity in all areas of the United States. In addition, it has not been sufficiently emphasized that even in areas known to be endemic the organism's distribution is spotty rather than continuous.[7, 9, 24] In a recent and more complete skin test survey in Pennsylvania, for example, Aronson and Taylor found a high rate of Histoplasmin sensitivity in the counties comprising the southeastern part of the state, in marked contrast to the low incidence ascribed to the state as a whole on the basis of previous, sporadic studies.[25] It was thus not surprising when the organism was subsequently isolated from a soil sample collected in Chester County, Pennsylvania, and from the native resident whose pulmonary infection was traced to exposure to that source.[23] Nor is it unlikely that there are other primary infections in that area which continue to be overlooked or attributed to other more usual causes. A similar situation undoubtedly exists in certain areas of New York where primary, pulmonary cases in residents exposed to a local focus of the organism were recently described.[3, 19]

The finding of an increased number of cases in any area is generally due to a greater awareness of the disease and improved diagnostic methods. Nevertheless, there are relatively new situations in which heterogenous populations rather than isolated groups might be expected to be exposed more frequently to this agent. Primary among these are the new suburban communities in various stages of development throughout the nation. These vast communities usually not only occupy territory that was farm land a short while before but are adjacent to others still used for this purpose. In many of these communities, gardening is a common pursuit. The readily accessible compost, as in this series of cases, is transported through densely populated areas in cars or trucks and, if contaminated, at least temporary foci are established in the vehicles, the routes they travel and the areas on which the compost is eventually spread The automobile which figured in this outbreak, for example, was traded for a new one within the week What of the new purchasers who were exposed to the spore laden trunk? And would such exposure constitute an unusual activity to which later infections might be traced?

Finally, there appears at the present time to be sufficient evidence to indict the chicken house, especially the old, unused one, as a prominent reservoir of this fungus, and possibly to use it as one of the indices as to whether histoplasmosis can be expected to occur in a community or not The close association of *H capsulatum* and soils enriched with excreta of chickens or other fowl was first pointed out by Zeidberg and Ajello in studies carried out in Tennessee , a curious predilection that has been amply verified by the extensive studies of Emmons as well as more limited ones by others in various eastern states[3, 5, 9, 19, 21, 23, 26] Our own experiences emphasize that it is the soil from the old and often abandoned coop, or areas on which such buildings have recently stood, that is particularly suspect These rich soils, in addition to having been undisturbed, usually are exposed to moisture but not to sun and thereby are ideal environments for the luxuriant growth of this organism[21, 27, 28] In the present study, it is to be noted that the organism was readily recovered from the source which met these requirements, but not from the garden which was continuously exposed to radiation The eastern seaboard states are densely populated with old towns and villages and the newer type of community described above All are heavily endowed with old chicken houses, many of which are no longer used It is perhaps time to question whether they are merely eyesores and as innocuous as they seem, and to regard them as potential reservoirs of *H capsulatum* regardless of their geographic area

SUMMARY

Four cases of primary pulmonary histoplasmosis occurred in a family living in a suburban housing development near Washington, D C Locating the exposure source of *H capsulatum* was complicated by the family's close association with a variety of possible foci These included decaying wood, birds, their nests and eggs, rodents domestic pets, and, a singular but unrelated incident involving a high concentration of dust During a follow up study 4½ years after the primary infections *H capsulatum* was isolated from the dirt floor of an old chicken coop on a nearby farm The father and son had collected chicken compost from this coop to fertilize the family garden about 10 days prior to onset of their infections The mother and sister in law worked in this garden during the next few days

Because of the increasing evidence that the District of Columbia area is one of high rather than low endemicity for this fungus, some of the limitations of the currently available map of distribution are pointed out The association of exposure to this organism with commonplace tasks, a possibly increasing

number of infections in heterogenous populations of suburban communities, and the prominence of the old chicken coop as a reservoir of *H. capsulatum* are also discussed.

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GENERALIZED EXCESSIVE OOZING IN PATIENTS UNDERGOING MAJOR SURGERY AND RECEIVING MULTIPLE BLOOD TRANSFUSIONS

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GENERALIZED oozing from small blood vessels in the operative field may occur during surgery. Usually it is short lived and minor in degree. On other occasions it may be excessive and prolonged or intractable. The cause is not well understood. Massive blood replacement,[1-4] a demonstrable circulating fibrinolytic enzyme,[5-11] hypo- or afibrinogenemia, and platelet deficiencies have all been suggested as the cause.[10-13]

A number of factors concerned with blood clotting and fibrinolysis have been measured before, during, and after surgery. An attempt has been made to correlate changes in these factors both with the occurrence of excessive oozing during surgery and with the number of transfusions administered.

TABLE I. INCIDENCE OF OOZING AND FIBRINOLYSIS IN RELATION TO SITE OF OPERATION

SITE OF SURGERY	TOTAL NUMBER PATIENTS	NUMBER SHOWING OOZING	NUMBER SHOWING	
			EUGLOBULIN ACTIVITY	WHOLE CLOT LYSIS
Head and neck	16	1	6	2
Thoracic	7	1	1	0
Abdomen and G. U.	48	9	13	5
Hepatic lobectomy	7	4	4	3
Total	78	15	24	10

MATERIAL AND METHODS

Most of the patients studied were undergoing extensive surgery for malignant neoplasms (Table I). Venous blood samples were drawn on the day before the operation, on

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TABLE II. PERTINENT DATA AND MAXIMUM CHANGES OBSERVED IN PATIENTS WITH EXCESSIVE SURGICAL BLEEDING

		AGE	SEX	SITE OF SURGERY	NO. UNITS BLOOD	EUGLOBULIN (HR.)	WHOLE BLOOD (HR.)	PROTHROMBIN TIME (SEC.)	PROTHROMBIN CONTENT (%)	FACTOR V (%)	FACTOR VII (%)	FIBRINOGEN (MG. %)	PROTHOMBIN CONS. (%)	PLATELET COUNT 10³/C.MM.	HYPOTENSION
	Series I														
1.	S. B.	60	M.	Laryngectomy and neck dissection	6	> 8 < 16*	—	17.2	90	78	—	160	> 90	205	No
2.	C. D.	33	F.	Anterior pelvic exenteration	7	1	3	17.1	52	54	—	160	> 90	112	No
3.	O. R.	41	F.	Cholangio-enterostomy	4	10	10	—	70	77	—	58	—	268	No
4.	P. S.	62	F.	Hysterectomy and node diss.	3	½	—	—	—	—	—	—	—	—	No
5.	C. W.	73	F.	Total pelvic exenteration	6	4	16	17.0	72	56	55	480	> 90	190	No
6.	C. S.	55	M.	Right hepatic lobectomy	15	1	2	18.9	69	42	75	< 100	> 90	183	No
7.	W. W.	48	M.	Right hepatic lobectomy	29	4	12	18.4	46	73	62	39	90	117	2 hr.
	Series II														
1.	C. H.	46	F.	Cholecystectomy	0	> 8 < 20*	> 24	14.9	60	71	100	440	32	—	No
2.	A. K.	68	M.	Pneumonectomy	4	⅔†	> 24†	19.0	86	42	59	345	> 90	250	No
3.	J. T.	48	M.	Laparotomy inoperable liver hemangioma	3	5‡	> 24‡	16.0	88	65	52	224	75	225	No
4.	T. A.	52	F.	Exenteration nephrectomy	11	> 24	> 24	15.2	70	61	100	375	78	112	No
5.	A. M.	57	F.	Hemipelvectomy	7	> 24	> 24	16.3	80	56	61	295	> 90	195	No
6.	M. R.	29	F.	Exenteration	8	> 24	> 24	15.3	59	60	78	375	90	295	No
7.	E. J.	60	F.	Right hepatic lobectomy; posterior gastrostomy	14	1	> 1 < 5½	17.5	70	40	59	340	> 90	150	1 hr.
8.	M. S.	49	F.	Right hepatic lobectomy	7	> 8 < 17*	> 24	19.8	62	—	52	422	—	240	No

*Since lysis may not have occurred within 10 hours, these patients are not considered to have had significant euglobulin fibrinolytic activity.

†Sample taken 40 minutes after closure. The previous sample was taken 2½ hours before the end of a 6 hour operation and did not show fibrinolytic activity. Oozing occurred 30 minutes before closure.

‡Sample taken 20 minutes after closure. Oozing occurred during closure. A sample taken 30 minutes before oozing was noted had no fibrinolytic activity.

the day of operation before and after the induction of anesthesia (thiopental, nitrous oxide, and ether), and at intervals during and after surgery. Fibrinogen and fibrinolysin determinations were done on all samples, and other studies outlined below were done in most instances.

The following determinations were made

1. Fibrinolytic Activity of Whole Blood The time required for complete lysis of a whole blood clot was determined at 37° C

2. Fibrinolytic Activity–Euglobulin Fraction A previously reported modification[14] of the method of Milstone[15] was used. In the early cases 50 units of thrombin were used but later this was changed to one unit of thrombin *

3. Fibrinogen: Quick's modification of the method of Cullen and Van Slyke.[16]

4. Platelet Count· This was performed on capillary blood[17] Normal values averaged about 250,000 per c.mm.

5. Prothrombin Time: Quick's method.[16] Normal values were less than 14 seconds

6, 7, and 8. Prothrombin, Factor V, and Factor VII Modifications of Owren's one stage assays were used.[18] Normal values were above 70 per cent

9. Prothrombin Consumption. Prothrombin determinations were carried out on serum from blood oxalated after one hour at 37° C[18] Normally, over 90 per cent of the plasma prothrombin was consumed.

10. Citrate Concentration: This was determined by the Natelson method[19] modified by Elliot[20]

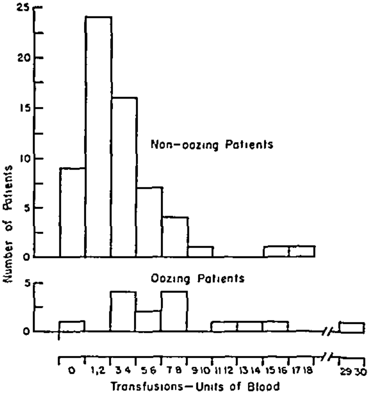

Fig 1—Number of transfusions given to nonoozing and oozing patients

RESULTS

Excessive generalized bleeding or oozing occurred in 15 of the 78 patients studied. It was observed during many types of operations but most frequently during hepatic lobectomy (Tables I and II).

Massive Blood Replacement and Changes in Platelet Count.—Four of the 15 oozing patients received over 10 units† of blood while only two of the 63 patients who did not ooze received this much blood (Fig. 1). A significant relationship between oozing and massive transfusion (Chi square = 6.36; P 0.02 = 5.41; P 0.01 = 6.64) is indicated, but it is impossible to prove that the multiple transfusions were not the effect of rather than the cause for the excessive bleeding.

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*Bovine Thrombin· Parke, Davis & Company, Detroit, Mich

†One unit is 600 ml A.C.D. bank blood, which contained 480 ml or 1 pint of blood and 120 ml of A.C.D. The average storage age of 295 units of bank blood given to 21 patients was 7 8 days.

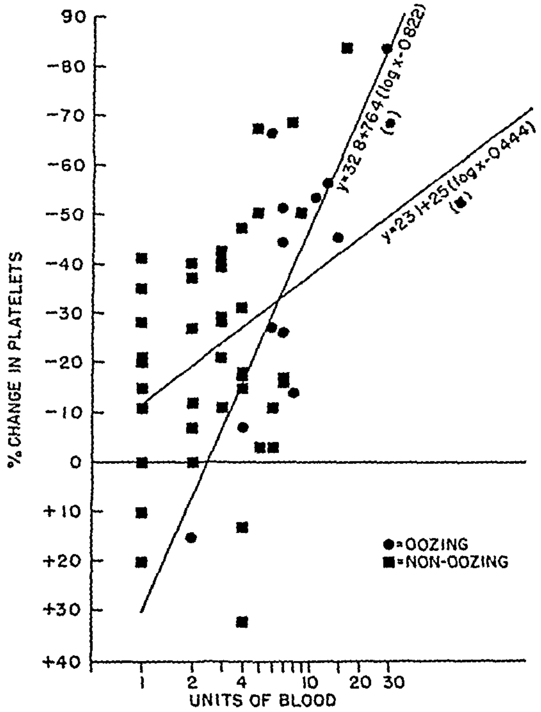

Fig. 2.—Relation between number of transfusions and per cent change in platelet count in oozing and nonoozing patients.

The platelet count after transfusions was compared with the preoperative count, and the per cent change plotted against the number of transfusions for 13 patients who oozed and 40 patients who did not ooze (Fig. 2). The average control platelet count was 329,000/c.mm. and was over 400,000/c.mm. in 25 per cent of the patients. The platelet count fell sharply after multiple transfusions. However, only two patients, neither of whom oozed, had platelet counts below 100,000/c.mm. after surgery and transfusions. When straight lines were fitted to the data by the method of least squares, there was a significant difference in slope between the oozing and the nonoozing patients (t = 4.68; t for P 0.01 = 2.68). This indicated that increasing amounts of blood caused a greater depression of platelets in oozers than in nonoozers. Despite this tendency, severe thrombocytopenia was not present in the oozing patients; the lowest platelet count in an oozing patient during surgery was 112,000/c.mm., and the average was 196,000 (Table II).

Plasma Citrate Concentration.—The plasma citrate concentration was increased in proportion to the amount and rate of blood replacement.[21] Although the plasma citrate level was elevated in all but two of the patients who oozed, it was not possible to establish a direct relationship between oozing and plasma citrate levels (Fig 3) Oozing did not always coincide with peak plasma citrate levels It did not always stop even when plasma citrate levels fell below 10 mg per cent, and sometimes ceased without an appreciable drop in the plasma citrate Plasma citrate levels of 30 or 40 mg per cent were not uncommon, and levels as high as 79 5 mg per cent were observed in the absence of oozing One gram of calcium, either as the chloride or gluconate was administered for each two units of transfused blood

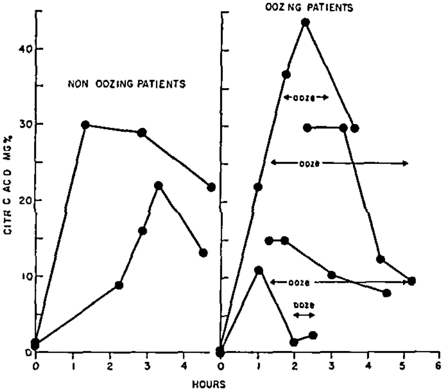

Fig 3—Plasma citrate concentration during surgery and multiple transfusions in selected nonoozing and oozing patients

Fibrinolytic Activity—Samples were considered to have activity only when complete lysis occurred in less than 24 hours for the whole blood samples, and in less than 10 hours for the euglobulin samples Euglobulin activity was frequently observed when a simultaneous sample did not show whole clot lysis When whole clot lysis was present, the euglobulin fraction always showed some fibrinolytic activity

The relationship between the type of operation and the occurrence of fibrinolytic activity is shown in Table I Patients undergoing hepatic lobectomy had the highest incidence of fibrinolytic activity

The 40 patients previously reported[22] are referred to as Series I and were studied between September, 1955, and March, 1956 None of these patients had fibrinolytic activity prior to surgery Seven of them oozed during surgery Whole clot lysis occurred in 5 of these oozing patients, but was not determined in the other 2 Six of the oozing patients had fibrinolytic activity of the euglobulin fraction (Fig 4, Table II) In contrast to the high incidence of fibrinolytic activity observed in the oozing patients, only 4 of the 33 nonoozing patients showed whole clot lysis, with lysis times between 8 and 24 hours. These 4 patients also showed euglobulin activity (Fig. 4).

The 38 patients studied from March to July, 1956, Series II, did not show as definite a relationship between oozing and fibrinolysis. Of the 8 oozing patients only one had whole clot lysis, and 3 showed definite euglobulin activity (Fig. 4, Table II). Of the 30 nonoozing patients, one had 2-hour whole clot lysis during surgery, 3 showed euglobulin activity prior to surgery, and 10 had euglobulin activity during surgery (Fig. 4). The techniques employed were the same in both series with the following exceptions. In Series II, blood samples were obtained less frequently prior to the oozing episode and in most patients samples were not obtained during the actual oozing. In Series I, the mixture of patients' euglobulin fraction and fibrinogen was clotted with 50 units of thrombin, whereas in Series II, only one unit of thrombin was used. This change was made when it was found that the thrombin was contaminated with bovine plasminogen.[23]

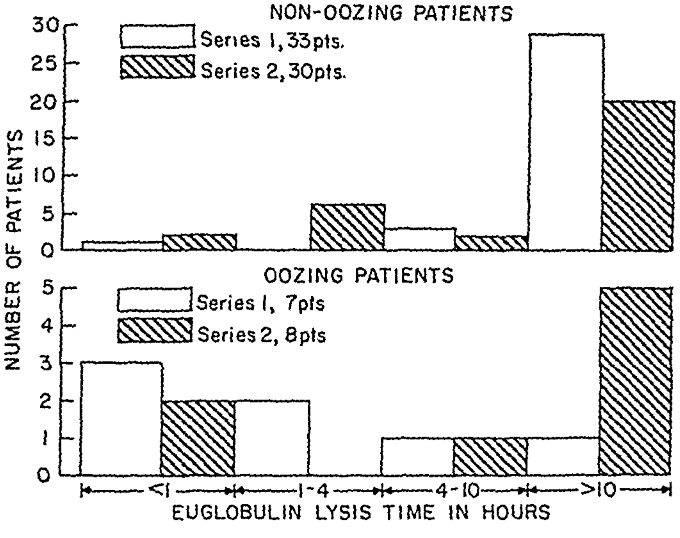

Fig. 4.—Euglobulin fibrinolytic activity in oozing and nonoozing patients.

When lytic activity was present, it was observed in one or more samples drawn close to the time of excessive bleeding. In 3 of the 9 oozing patients with significant fibrinolytic activity during surgery, no fibrinolytic activity was found in samples taken about one hour after closure. Two patients in Series II oozed just before or during closure, and no blood samples were taken until about an hour after closure. These postoperative samples showed fibrinolytic activity which was no longer present two hours later. Three patients who showed fibrinolytic activity while they were oozing during surgery, showed less activity in postoperative samples taken ½ to 3 hours after closure, and activity was no longer present in subsequent samples taken 3 to 6 hours later. In these patients, no apparent oozing occurred after closure. In one patient

(C D), oozing persisted for several hours after operation Four hours post operatively this patient showed fibrinolytic activity which was more marked than during surgery Two hours later fibrinolytic activity had disappeared and bleeding was no longer observed

Fibrinogen—The fibrinogen concentration was below 170 mg per cent in 5 and below 100 mg per cent in 3 of the oozing patients Two of the latter had had hepatic lobectomies (Table II) It is of interest that all of these were in Series I All 5 had some fibrinolytic activity It seems probable that a relationship exists between the two Hypofibrinogenemia also developed in 4 nonoozing patients * In one of them, a patient undergoing hepatic lobectomy, fibrinogen concentration fell below 100 mg per cent, and in the other 3, who were undergoing other operations, it fell to slightly below 150 mg per cent Fibrinolytic activity was not detected in the latter 3 patients

Several patients showed marked decreases in fibrinogen concentration during surgery but because the control concentration was high it did not fall to levels usually associated with bleeding Fibrinogen fell more than 100 mg per cent during surgery in patients who had no demonstrable fibrinolytic activity as well as in those with fibrinolytic activity In some patients, the fall in fibrinogen could be attributed to transfusions, in which the patient's blood, with an abnormally high fibrinogen concentration, was replaced with bank blood containing a normal level of fibrinogen

Prothrombin Time and Factors Influencing It—The longest prothrombin time among the nonoozing patients was 18 8 seconds, compared to 19 0 seconds among the nonoozing patients Reduction of prothrombin concentration was almost uniformly observed among the patients undergoing hepatic lobectomy, but values below 46 per cent were not reached until after surgery had been completed[33] None of the other operative procedures resulted in less than 50 per cent prothrombin concentration The proportion of patients with low prothrombin concentration was about equal in the oozing and nonoozing groups Factor VII was not measured in all patients but, when determined, showed similar changes

Factor V was low among the oozing patients more often than prothrombin Furthermore, a decrease in factor V was found with significantly greater frequency among the oozing patients than among those who did not bleed excessively (Chi square = 14 3, P 0 01 = 6 6) Fibrinolytic activity was present in all but one (T A) of the oozing patients who showed a drop in factor V and may have been responsible for destruction of this clotting factor[31]

Prothrombin Consumption—Three of the oozing patients (two of them women) showed less than 90 per cent consumption of prothrombin in one hour in samples obtained immediately prior to or early during surgery, although they had adequate platelet counts It is possible that this abnormality accounts for the excessive surgical bleeding seen in these patients[25]

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*Fourteen patients are not included because determinations were made with the Parfentjev method[24] When the fibrinogen concentration is low this method gives results considerably below those obtained by the Cullen and Van Slyke method

Shock, Transfusion Reactions, and Dextran Administration.—Shock and tissue hypoxia may cause derangements in the hemostatic mechanism either during operation or in the postoperative period. In this series there were 5 patients in frank shock. Only two of them oozed and had fibrinolytic activity. In the remaining 13 patients who exhibited oozing, significant hypotension during the operation or the postoperative period was not noted.

Although hemolytic transfusion reactions have been implicated as a cause of excessive surgical bleeding,[26] there is no evidence that any of our patients received incompatible blood. To eliminate subgroup incompatibility, direct cross-matching as well as indirect Coombs tests were done on all patients.

Two of the oozing patients received dextran during surgery. Although this plasma volume expander has been found to induce a hemorrhagic syndrome when given in large amounts to normal individuals,[27] dextran was not responsible for the initiation of excessive bleeding in our patients, since oozing was present before dextran was given.

TABLE III. CHANGES SHOWN BY NONOOZING AND OOZING PATIENTS

	NO CHANGE	INADEQUATE DATA	CHANGE IN SINGLE CATEGORY			
			LYSIS*	PROTHROM. FACTORS†	PLATS‡	TOTAL
Nonoozing	21	7	10	20	1	31
Oozing	1	1	0	3	0	3

Changes in Multiple Categories

	LYSIS FIBRIN.§	LYSIS PROTHROM. FACTORS	LYSIS FIBRIN. PROTHROM. FACTORS	LYSIS PROTHROM. CONS.‖ PROTHROM. FACTORS	PROTHROM. FACTORS PROTHROM. CONS.	PLATS. PROTHROM. FACTORS	TOTAL
Nonoozing	0	2	1	0	0	1	4
Oozing	1	4	2	1	2	0	10

*Euglobulin lysis time less than 10 hours.
†Prothrombin, factor V or VII below 70 per cent.
‡Platelet count under 100,000 c.mm.
§Less than 100 mg. per cent.
‖Less than 90 per cent in one hour.

Differences Between Oozing and Nonoozing Patients.—The results are summarized in Table III. The changes observed were classified in the following categories: (1) euglobulin fibrinolytic activity with lysis in less than 10 hours; (2) prothrombin, factor V, or factor VII below 70 per cent of normal; (3) fibrinogen concentration less than 100 mg. per cent; (4) platelet count less than 100,000/c.mm.; and (5) prothrombin consumption less than 90 per cent in one hour. The patients are divided into four groups: those showing no changes; those with incomplete data; those showing only one type of deficiency, that is, a change in only one category; and finally, patients showing multiple deficiencies or changes in more than one category. Among the nonoozing patients with adequate data, 21 showed no change in any of the factors studied, 31 showed changes in only a single category, and only 4 showed multiple changes. In contrast, only one of the oozing patients showed no change in any category, 4 showed a change in a single category only, and 9 showed changes in more than one category If the 3 oozing patients with poor prothrombin consumption are omitted because this alone is presumably an adequate explanation of their bleeding, 3 oozing patients showed a change in a single category only and 7 showed multiple changes

There proved to be *significant differences between the distribution of oozers* and nonoozers in the 3 categories (no change, single change, and multiple changes) (Chi square = 18 86, P 0 01 = 9 21) The significance becomes even greater if the oozing patients with poor prothrombin consumption are included Many more oozers are found in the multiple change category than one would expect if the distribution were the same for oozers and nonoozers When a comparison is made between patients who had multiple changes and those who had changes in one category only, there is also a significant difference *(Chi square = 11 43, P 0 01 = 6 64) Those patients who had a change in* more than one category showed a significantly greater tendency to ooze

DISCUSSION

Although the occurrence of fatal hemorrhage in obstetrics and surgery is rare, episodes of excessive bleeding during massive surgery are not uncommon In this series, oozing occurred in 15 of 78 patients Only 4 of the oozing patients received over 10 units of blood and in one of these the transfusions were *the result rather than the cause of the excessive bleeding Hence, massive* transfusion was not a cause of oozing in most of the patients

Thrombocytopenia following massive blood replacement has been reported by Stefanini and associates[1] and by Krevans and Jackson [3] We have observed profound falls in the platelet count after less than 8 units of blood, and always after the administration of 10 or more units The percentage reduction in count in our patients is approximately the same as in the patients studied by Krevans and Jackson [3] However, only two of our patients had platelet counts below *100,000/c* mm after transfusion One explanation for the lack of thrombocytopenia in our cases is the high pretransfusion platelet count in many of our patients Another is that Krevans and Jackson reported the lowest platelet count within 48 hours after transfusion, whereas we report the count within a few hours The platelet count generally continues to fall for one or two days after blood replacement Thrombocytopenia did not appear to be a significant factor in the etiology of oozing in our series, although there was a tendency for a greater depression of the platelet count among the oozers

Although "citrate intoxication" has been discussed repeatedly since the advent of the modern blood bank, there has been little or no clinical study of the correlation between blood citrate levels and oozing In this series, patients showed an oozing tendency with and without elevated blood citrate levels Some patients with markedly elevated citrate levels did not ooze There was no evidence from our studies that elevated citrate concentration affected blood clotting Presumably, the administration of calcium salts and the mobilization of body calcium stores adequately maintained ionized calcium

Our results suggest that two criteria should be observed in an attempt to assess the relationship between fibrinolytic activity and excessive surgical bleeding. First, samples should be drawn during the period of active bleeding, since fibrinolytic activity often disappears when oozing ceases. Second, frequent samples should be obtained. Because of the rapid fluctuations in fibrinolytic activity, a single sample may give misleading results.

The mechanisms which activate the fibrinolytic enzyme are not clearly understood. Stress,[28] shock,[29] and hypoxia[22] can activate fibrinolysin and may play a role in surgical cases.[28] Shock was not severe in most of our oozing patients. Hypoxia was considered to be the cause of fibrinolytic activity in two of the nonoozing patients in the first series.[22] Tissues, particularly the lung and uterus, are said to contain activators[30] which may be released into the circulation during surgical trauma. Fibrinolysin may interfere with hemostasis by lysis of clots or it may destroy some of the circulating blood clotting factors.[31]

Previous studies of surgical bleeding have consisted, for the most part, of case reports of patients with very severe hemorrhagic syndromes. A striking feature of some of these reports has been the failure of the patient's blood to clot even when thrombin was added.[5, 6, 10] This has been attributed to the absence of circulating fibrinogen. It should be remembered that incoagulable blood may result from rapid lysis of newly formed fibrin strands. In vitro lysis of fibrin occurs more rapidly than does destruction of the fibrinogen in blood prevented from clotting by citrate or oxalate, so that rapid whole clot lysis may occur when the fibrinogen concentration is only moderately low. For example, in one sample in a severely oozing patient not included in this series, the clot had lysed completely in less than 20 minutes. If inadequately observed, this might have been mistaken for complete absence of clot formation and interpreted as afibrinogenemia; yet, a simultaneous oxalated sample had a fibrinogen concentration of at least 175 mg. per cent.

Fibrinogen concentrations below 100 mg. per cent were observed in only 3 of the oozing patients, all of whom had fibrinolytic activity. In cases reported by others, hypofibrinogenemia in surgery or obstetrics was often associated with demonstrable fibrinolytic enzyme, but, in some, fibrinolytic activity was not detected.[12, 13] In the latter cases, intravascular coagulation has been implicated as a cause of hypofibrinogenemia.[12, 32] The failure to detect activity could be ascribed to lack of sensitivity of the method employed,[12] or to disappearance of activity at the time of sampling.

Low fibrinogen concentration, rather than complete afibrinogenemia has been observed in most of the bleeding patients described by Coon and Hodgson,[9] Hodgkinson and associates,[10] and Phillips, Rowley, and Habif.[11] Although patients with congenital afibrinogenemia have fairly adequate hemostasis in the face of day-to-day trauma,[25] Phillips and associates[11] observed operative bleeding in patients with concentrations of fibrinogen as high as 125 to 175 mg. per cent and suggest that a higher level of fibrinogen is needed for hemostasis during surgery.

Decreased concentrations of prothrombin, factor V, and factor VII were found during surgery in patients who did not ooze, as well as in some who had excessive bleeding These factors may have been destroyed by the fibrinolytic enzyme in some cases, but similar reductions were observed in patients in whom fibrinolytic activity was not detected Since these clotting factors are short lived,[32] this fall in concentration may represent utilization without adequate replacement The liver is necessary for normal production of prothrombin and factors V and VII With partial hepatectomy, factor V reaches its lowest level within 12 hours after surgery, prothrombin and factor VII fall more slowly, but are generally already somewhat reduced by the end of surgery[33] It is possible that the minor decreases in these factors observed in some patients undergoing operations other than hepatic lobectomy may be the result of a temporary decrease in hepatic production

We conclude that no single factor is responsible for excessive bleeding during surgery In fact, there was a statistically significant relationship between the occurrence of excessive bleeding and the presence of multiple changes in the factors studied Fibrinolytic activity was the change most frequently associated with oozing, but fibrinolysis was sometimes present without obvious oozing and was occasionally absent in oozers In most of the patients who oozed excessively, some change in addition to fibrinolysis was noted, usually a decrease in fibrinogen, prothrombin, factor V, or factor VII Although these clotting factors may have been destroyed by the fibrinolytic enzyme, similar decreases were observed in patients without detectable fibrinolytic activity

Multiple deficiencies were also found in two patients with severe oozing, not included in the series because of lack of control data Both whole blood and euglobulin fibrinolytic activity were very pronounced in these patients The fibrinogen level was low normal, but prothrombin, factor V, and factor VII, and platelet levels were below normal

It is difficult to assess therapeutic measures because both lysis and oozing may cease without treatment We have used lyophilized fresh (antihemophilic) plasma largely because many of the samples tested had very high antifibrinolytic activity It also contains prothrombin, factor VII, and fibrinogen in approximately normal concentration, but there is a subnormal concentration of factor V Fresh whole blood collected in plastic bags is recommended when thrombocytopenia is present, and fibrinogen (fraction I) should be given when severe hypofibrinogenemia occurs

SUMMARY

Blood clotting factors and fibrinolysis were studied in 78 patients before, during, and after major surgery

Fifteen patients oozed excessively during surgery Oozing could not be ascribed to massive blood replacement, and in none of the oozing patients was the platelet count below 100,000/c mm Plasma citrate concentration bore no relationship to the occurrence of oozing

Fibrinolytic activity of the euglobulin fraction was found during or shortly after surgery in nine of the 15 oozing patients Active whole clot lysis was

observed in 6 cases. Fourteen of the 63 nonoozing patients had euglobulin fibrinolytic activity and 5 had whole clot lysis. Fibrinogen concentration fell below 100 mg. per cent in only 3 of the oozing patients, 2 of whom were undergoing hepatic lobectomy. Decreases in prothrombin and factor VII concentrations during surgery occurred with equal frequency among oozers and nonoozers, whereas decreases in factor V occurred more frequently in oozing patients. Values below 50 per cent of normal were only observed in patients undergoing hepatic lobectomy.

All but one of 14 oozing patients showed a change in at least one of the factors measured. Three had poor prothrombin consumption before surgery, 3 had prothrombin or factor V concentrations below 70 per cent, and the remaining 7 had fibrinolytic activity plus some other change. The correlation between oozing and the occurrence of multiple changes was statistically significant.

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STUDIES IN IRON TRANSPORTATION AND METABOLISM

X. LONG-TERM IRON OVERLOAD IN DOGS

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INTRODUCTION

HEMOCHROMATOSIS, characterized by pigmentary cirrhosis, pancreatic fibrosis with diabetes, and extensive parenchymal organ infiltration with iron, has not been produced in experimental animals despite attempts by many investigators. In these unsuccessful efforts a wide variety of animal species, many forms of iron compounds, and multiple routes of administration have been used. Many attempts to produce hemochromatosis experimentally may have failed because the amounts of iron given were too small or the subsequent period of observation too brief to allow the changes of this disease to occur. However, patients given multiple transfusions[1, 2] or prolonged excessive oral doses of medicinal iron,[3-6] and Bantu natives[7-10] ingesting large amounts of dietary iron do develop hemosiderosis and may show cirrhosis of the liver and rarely diabetes. That conditions other than idiopathic hemochromatosis are associated with increased tissue iron stores and organ damage in human beings, makes more remarkable the failure to produce similar changes in animals. An examination of previous experimental attempts to produce hemochromatosis seems indicated in order to view the problem more clearly and to evaluate the results we shall present in this study.

REVIEW OF LITERATURE

Among the most complete studies to delineate the pathogenesis of hemochromatosis were those of Polson,[11-13] who conducted both acute and long-term experiments on the fate of colloidal iron administered intravenously to rabbits. Using relatively small doses of iron (0.1 to 0.3 Gm./Kg.) and observing the animals for only a year, he found the injected iron was deposited initially in largest amounts in the reticuloendothelial elements of liver, spleen, lymph nodes, and bone marrow. Small amounts of hemosiderin were present as emboli in pulmonary and glomerular capillaries and in scattered macrophages elsewhere in the body. Little iron was found in hepatic parenchymal cells until redistribution from Kupffer cells occurred 6 to 8 months after the beginning of injections. Though considerable aggregation of iron granules occurred, there was little cellular reaction to its presence, and nothing

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similar to the fibrosis and cirrhosis of hemochromatosis was produced This same investigator injected colloidal iron in doses of 0 13 to 0 33 Gm /Kg subcutaneously and intraperitoneally into rabbits for periods as long as 4 years with essentially the same histologic results

Careful experiments similar to those of Polson were reported in 1930 by Cappell[14] who injected saccharated iron oxide in 550 mg /Kg doses into mice and rats which were thereafter observed for periods as long as 14 months With minor exceptions, his results confirmed Polson's work and led him to conclude that "nothing analogous to the lesions of hemochromatosis in the human subject has been observed in the organs of animals subjected to excess iron over prolonged periods "

Andersson[15] injected a preparation similar to saccharated iron oxide intravenously into rabbits and followed 4 animals for as long as one year Doses of iron in the range of 0 3 to 0 35 Gm /Kg produced no cirrhosis or other organ damage

Extensive studies using intravenously administered saccharated iron oxide or subcutaneous and intraperitoneal injections of "ferric hydroxide ferrous ascorbate" in mice, rats, rabbits, and guinea pigs were performed by Nissim[16] He achieved doses of iron as high as 2 16 Gm /Kg in mice and observed them until death occurred ' months ' after the last injection Guinea pigs were followed for approximately 3 months from the beginning of subcutaneous injections of "ferric hydroxide ferrous ascorbate" in doses as high as 1 Gm of iron per kilogram of body weight While tissue damage was produced as evidenced by hemorrhagic embolic phenomena in the lungs, patchy parenchymal damage in the liver, and adrenal hemorrhages no cirrhosis of the liver or pancreatic fibrosis was observed This author emphasized that the distribution of iron deposits achieved and the organ damage produced depended upon the type of preparation used

In as yet unpublished observations on the long term toxicity of an iron dextran complex Golberg[17] reports no evidence of cirrhosis or organ damage from iron deposits resulting from doses as high as 1 65 Gm /Kg in rats or rabbits observed more than 40 weeks

Hemoglobin iron liberated from the animal s own red cells by hemolytic agents[18 24] or from nonviable transfused cells has been used by several investigators in attempts to produce hemochromatosis in experimental animals Among the earliest of these studies were those of Rous and Oliver[25] who transfused rabbits with citrated whole blood daily for periods up to 6½ months They produced abundant hemosiderin pigmentation of reticuloendothelial cells but no cirrhosis their conclusion was "that hemosiderin, when enclosed in living cells, has no noteworthy stimulative effect on connective tissue "

Since the excess iron in idiopathic hemochromatosis and in cytosiderosis of Bantu natives must ultimately be derived from increased absorption from the gastrointestinal tract,[9 10 26 27] many investigators have attempted to increase iron absorption in animals While it has been repeatedly shown that some form of block to uncontrolled iron absorption exists in several animal species as well as in man, Hegsted and co workers[28] demonstrated in rats, mice, guinea pigs, and chickens that if sufficient iron is added to a normal diet all species will absorb large amounts of iron They also showed[29 30] that even greater amounts of iron will be absorbed if a corn grit diet low in phosphorus or high in certain amino acids is fed When one increases the absorption of iron by these means, iron is deposited primarily in the liver and spleen Its distribution in parenchymal as well as in reticuloendothelial cells is somewhat suggestive of that in hemochromatosis, but there is no associated *fibrosis or cirrhosis Wyatt and Howell*[31] *extended the corn grit dietary experiment* of Hegsted and co workers and added other forms of tissue injury produced by castration or feeding of copper salts Excessive iron storage in the liver was again produced, but no evidence of progressive parenchymal fibrosis was observed Likewise, Rather[79] fed ferric citrate alone or combined with a low protein diet to rats observed as long as 20 months He found no fibrosis of the liver or pancreas despite large hepatic hemosiderin deposits

Increased absorption of iron added to the diets of golden hamsters has been described[32] when emulsifying agents are also added Siderosis of the liver is thereby produced, but no convincing evidence of cirrhosis or other changes of hemochromatosis have been described

Following ligation of the pancreatic ducts in cats, fibrosis of the pancreas and siderosis of the liver, spleen, and other organs occurs as first described by Taylor, Stiven, and Reid[33]

TABLE IA. ATTEMPTS TO PRODUCE IRON OVERLOAD IN EXPERIMENTAL ANIMALS. IRON INTRODUCED PARENTERALLY

AUTHOR	EXPERIMENTAL ANIMAL	ROUTE OF ADMINISTRATION	Fe COMPOUND AND DOSE	DURATION OF OBSERVATIONS	COMMENT
Polson[11-13]	Rabbit	Intravenous	Colloidal Iron (0.1-0.3 Gm./Kg.)	14 mo.	Iron was confined initially to reticuloendothelial cells with eventual redistribution to hepatic parenchymal cells (after 6-8 mo.) without fibrosis or cirrhosis
	Rabbit	Intramuscular Intraperitoneal	Colloidal Iron (0.13-0.33 Gm./Kg.)	4 yr.	Essentially the same results as with intravenous administration
Cappell[14]	Mouse Rat	Intravenous	Saccharated Iron Oxide (0.55 Gm./Kg.)	14 mo.	Confirmed Polson's observations
Andersson[15]	Rabbit	Intravenous	Saccharated Iron Oxide (0.3-0.35 Gm./Kg.)	1 yr.	Confirmed Polson's observations
Nissim[16]	Mouse	Intravenous	Saccharated Iron Oxide (1.8-2.16 Gm./Kg.)	5 mo.	
	Rat	Intravenous	Saccharated Iron Oxide (0.6-1.0 Gm./Kg.)	6-12 wk.	Hemorrhagic embolic phenomena in the lungs, patchy parenchymal damage in the liver, and adrenal hemorrhages were produced. However, no cirrhosis of the liver or pancreatic fibrosis was seen
	Rabbit	Intravenous	Saccharated Iron Oxide (0.09-0.54 Gm./Kg.)	4-6 wk.	
	Guinea pig	Intraperitoneal and subcutaneous	Ferric hydroxide Ferrous Ascorbate (0.17-1.0 Gm./Kg.)	3 mo.	
Golberg[17]	Rat Rabbit	Intramuscular	Iron Dextran (1.65 Gm./Kg.)	10 mo.	No evidence of cirrhosis or organ damage despite massive hemosiderosis
Finch[42, 43]	Dog	Intravenous	Iron Ascorbate Gelatin (1.44 to 4.20 Gm. Fe)	2 yr.	No cirrhosis or organ damage
	Dog	Intravenous	Red Blood Cells (1.76 Gm. Fe)	15 mo.	Iron was deposited mainly in the reticuloendothelial cells; the distribution was comparable to that in dogs given iron ascorbate gelatin. No tissue damage
	Dog	Intravenous	Saccharated Iron Oxide (2 Gm./Kg.)	2 yr.	Dogs developed hepatomegaly, persistent ascites, and "other evidence of liver failure" but minimal fibrosis
Rous and Oliver[25]	Rabbit	Intravenous	Red Blood Cells (Approx. 1 Gm. Fe)	3-6½ mo.	Transfused citrated whole blood and produced abundant hemosiderin pigmentation of reticuloendothelial cells but no cirrhosis
Krumbhaar and Chanutin[44]	Dog	Intravenous	Red Blood Cells (2.5-5.0 Gm. Fe)	6 mo.	Transfused whole citrated blood (5-10 L.) and produced hemosiderosis confined mainly to the reticuloendothelial cells without fibrosis
Platzer and Young[2]	Dog	Intravenous	Red Blood Cells (0.14-0.22 Gm./Kg.)	3-11 mo.	Produced hemosiderosis without fibrosis or other abnormalities

TABLE IB ATTEMPTS TO PRODUCE IRON OVERLOAD IN EXPERIMENTAL ANIMALS
IRON INTRODUCED ORALLY

AUTHOR	EXPERIMENTAL ANIMAL	ROUTE OF ADMINISTRATION	Fe COMPOUND AND DOSE	DURATION OF OBSERVATIONS	COMMENT
Hegsted et al.[28]	Rat Mouse Chicken Guinea pig	Oral	2 12% Fe citrate alone or with corn grits	27 81 days	When sufficient iron was added to a normal diet increased absorption occurred Even greater absorption occurred if corn grit diet low in phosphorus was fed
Wyatt and Howell[31]	Rat	Oral	2% Fe citrate *plus corn grits*	50 150 days	Extended corn grit diet experiments of Hegsted and added tissue injury in the form of castration and feeding copper salts Produced increased hepatic iron storage without fibrosis
Rather[79]	Rat	Oral	6% Fe citrate alone or with a low protein diet	20 mo	Produced large amounts of hemosiderin in the liver without fibrosis of liver or pancreas
Finch[42]	Dog	Oral	2% Fe citrate plus corn grits	2 6 mo	Produced marked hepatic parenchymal hemosiderosis No cirrhosis of the liver, no pancreatic or splenic fibrosis
Wissler et al[32]	Hamster	Oral	--	2 5 mo.	Polyoxyethylene sorbitan monolaurate (Tween 20) given orally produced increased iron absorption but no changes of hemochromatosis
Taylor et al[33]	Cat	Oral	--	55 199 days	Ligation of pancreatic ducts led to pancreatic fibrosis and increased iron absorption Hemosiderosis was accentuated by dietary iron supplements No hemochromatosis seen
Gilman et al.[34]	Cat	Oral	--	--	Repeated and confirmed the observations of Taylor et al
Kinney et al[45]	Dog	Oral	--	2 5 mo	Confirmed observations of Taylor et al in dogs No evidence of cirrhosis of the liver or hemochromatosis
Kinney et al[35]	Rat Mouse	Oral	2% Fe citrate plus DL ethionine	28 days	Ethionine produced damage to the pancreas, liver, and testes plus increased iron absorption When iron was added to the diet, lesions superficially like those of hemochromatosis appeared
Hall and MacKay[36]	Rabbit	--	--	21 105 days	Copper acetate given by mouth produced hepatic necrosis and eventual cirrhosis plus hemofuscin deposition with slight degrees of hemosiderosis
Mallory and Parker[37]	Rabbit	--	--	5 mo.	Similar experiments to Hall and MacKay's using subcutaneous injections of copper
Herbut et al[41]	Rabbit	--	--	14 102 days	Alloxan produced pancreatic and hepatic necrosis with eventual fibrosis plus slight hemosiderosis of liver and spleen

and confirmed by the Gillmans.[31] The siderosis was further accentuated by feeding a diet high in iron. Except for the duct-ligated pancreas, no fibrosis accompanied the iron so deposited in the tissues.

Ethionine administration produces damage to the pancreas, liver, and testes plus increased iron absorption. When iron is added to the diets of rats or mice so treated, siderosis of the liver and other organs plus the ethionine-induced damage produces lesions superficially like those of hemochromatosis.[35] Likewise, copper given by mouth[36] or subcutaneously[37] to rabbits results in necrosis and eventual cirrhosis of the liver with slight degrees of siderosis. While disturbances in copper metabolism have been described in idiopathic hemochromatosis[38-40] there is little evidence to suggest that copper plays an etiologic role in its production. Similarly, the toxic effects of alloxan in producing pancreatic and hepatic necrosis with eventual fibrosis and slight hemosiderin deposition have occasioned the use of this drug in unsuccessful attempts to produce hemochromatosis in rabbits.[41]

Most of the above-mentioned techniques have also been applied in attempts to produce hemochromatosis in the dog. In a comprehensive study of iron storage, Finch[42] injected 1.44 to 4.2 Gm. of iron as iron-ascorbate gelatin intravenously into 4 dogs which were then observed for periods as long as 2 years. Despite increases of iron concentration in the liver to approximately 25 times normal, no cirrhosis or other organ damage was produced. Transfusions of nonviable red cells to a total of 1.76 Gm. of iron were given to another dog sacrificed after 15 months. The distribution of iron from hemoglobin breakdown was entirely comparable to that produced by the iron-ascorbate gelatin and no tissue damage was mentioned other than scars involving wedges of renal tubular cells. Five dogs were fed a corn-grit diet supplemented by 2 per cent ferric citrate for periods of from 60 to 175 days. As had been demonstrated in rats and mice on a similar diet, iron concentrations in the livers of these dogs increased on the average to approximately 25 times normal while in the spleens there was a tenfold increase. Distribution of iron tended to be greater in parenchymal cells here than in dogs given iron parenterally, though reticuloendothelial deposits were also great. Again, no evidence of cirrhosis of the liver or fibrosis of pancreas or spleen was found. In a subsequent paper Finch[43] cites his experience with the intravenous injection of 2 Gm. of iron per kilogram of weight as saccharated iron oxide into normal dogs which were observed for two years. These animals developed hepatomegaly, persistent ascites, and ''other evidence of liver failure,'' but minimal fibrosis in proximity to siderotic nodules in the liver was the only change resembling the cirrhosis of idiopathic hemochromatosis.

Transfusions of citrated whole blood totaling 5 to 10 L. were given to dogs over a 6-month period by Krumbhaar and Chanutin.[44] When sacrificed, the animals showed marked congestion of all organs with hemosiderin pigmentation confined to macrophages in liver, spleen, lymph nodes, and other organs; there was no fibrosis. Likewise, Platzer and associates[2] transfused two dogs to iron levels of 0.14 and 0.22 Gm./Kg. before sacrifice at 3 and 11 months. They reported that with the exception of the polygonal cells of the liver, all visible iron was stored in reticuloendothelial cells, and neither fibrosis nor other abnormalities were noted in any organ. Pancreatic duct ligation in dogs[45] resulted in moderate siderosis of liver and spleen after 5 months but did not produce cirrhosis or other changes of hemochromatosis. These observations are summarized in Tables Ia and Ib.

The experimental attempts to produce hemochromatosis in animals summarized above clearly point up the relative ease with which iron can be introduced into the organism as well as the great difficulty of achieving parenchymal localization of the iron and of producing the fibrosis of liver, spleen, and pancreas which are hallmarks of idiopathic hemochromatosis in human beings. In many instances the concentration of iron achieved seemed too small or the period of observation too brief to be sure that the lesions of this disease had time to appear. With these objections in mind the following studies were undertaken.

Normal dogs were given iron as saccharated iron oxide or as hemoglobin from transfused red blood cells in doses of 0.5 to 1.0 Gm./Kg. body weight. These dogs were then observed for periods as long as 7 years During the period of observation, measurements of hepatic function were performed together with evaluation of hematologic status and changes in iron metabolism Repeated biopsies of liver, spleen, pancreas, and other organs were made for comparison with the histologic findings at autopsy by these means the initial deposition, redistribution, and cellular reaction to iron deposits were studied Chemical estimates of the iron content of various tissues were performed to quantitate iron deposition Despite concentrations of iron chosen to correspond to those found in human hemochromatosis, no findings suggestive of this disease were detectable even after 7 years Our failure to produce hemochromatosis in dogs, plus the similar experience of many investigators using other animals, raises the question whether increased iron deposits by themselves eventually produce the changes of hemochromatosis or whether additional factors must be present This question is considered in the following discussion, though no definite conclusions can be achieved with the information now available

MATERIALS AND METHODS

Healthy, full grown, mongrel dogs weighing 5 9 to 17 5 kilograms were used The animals were housed in individual cages, and were fed commercial dog chow During an initial period of observation the animals were given hexylresorcinol as a vermifuge At intervals throughout the study the feces were tested for occult blood by Gregerson s benzidine method[46] and whenever blood was found the vermifuge treatment was repeated Weights were obtained at frequent intervals

Red cell counts were made at weekly intervals at first, and less frequently during the later phases of the study Trenner red cell pipettes and counting chambers certified by the United States Bureau of Standards were used Initially hemoglobin was determined by the oxyhemoglobin method described by Evelyn[47] and in the last year of studies by the cyanmethemoglobin technique[48] Packed red cell volume was determined from arterial or venous blood Serum iron was measured by a method previously described[49] The iron binding capacity of serum was measured by the method of Rath and Finch[50] or by that of Ventura[51] Bromsulphalein retention as described by Gaebler[52] and serum bilirubin by Watson's modification of Evelyn and Malloy's method[53] were determined at 2 week intervals for 3 months and less frequently thereafter Fractional serum protein determinations were performed by Weichselbaum s method[54] Several specimens of serum were analyzed by paper electrophoretic methods[55] and scanned with a Spinco Analytrol Fasting blood sugar determinations were done by a modified Nelson technique[56] at approximately monthly intervals for 6 months, then less frequently Oral glucose tolerance tests as done by Fajans and associates[57] were performed on 3 animals shortly before sacrifice Absorption of radioactive iron was measured in 3 dogs[58] The utilization of intravenous tracer doses of radioactive iron was also studied[59]

A single lot of saccharated iron oxide* was injected intravenously into 4 dogs over a 6 to 10 week period In order to avoid perivenous infiltration of the iron solution the injection was begun with isotonic saline, which was allowed to flow into the saphenous vein from a small elevated reservoir As soon as the saline was flowing freely, the desired amount of Ferrivenin (5 to 15 ml containing 100 to 300 mg of iron) was added The last traces were washed into the vein by addition of saline to the reservoir Injections were given 5 times a week until a level of 0 5 Gm or 1 0 Gm/Kg was reached

Five dogs received transfusions of blood from compatible donors (determined by cross matching initially and after the third transfusion) At first the dogs were given washed red cells suspended in saline to about half the original whole blood volume After the sudden death of 3 of the dogs whole blood was substituted for washed red cells because death was believed to have been due to a rapid increase in concentration of erythrocytes in the circulation as the saline diffused out. When whole blood transfusions were substituted, sodium citrate rather than A.C.D. solution was used as a preservative and the blood was stored for 7 days before transfusion. These measures allowed more rapid red cell breakdown and thereby relieved the load on the recipient's circulation. As a further precaution, transfusions of 200 ml. of whole blood were given 3 times a week rather than a transfusion of the washed cells from 800 ml. of blood once a week.

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*Ferrivenin Lot No 10369 Benger Laboratories Limited Holmes Chapel Cheshire England

Transfusions to the two surviving dogs were continued for 6 to 10 months until an iron concentration of 0.5 Gm. per kilogram of body weight was attained. This iron level and those achieved with saccharated iron oxide were chosen to correspond to the concentrations of iron in patients with idiopathic hemochromatosis.

Laparotomies were performed under Nembutal anesthesia on 6 dogs before beginning iron injections. Wedge biopsies were obtained of liver, spleen, pancreas, kidney, a mesenteric lymph node, muscle, and skin. Measurement of the iron concentration of the liver section and microscopic examination of all these tissues were made. Within a month after completion of Ferrivenin injections a skin biopsy for iron analysis and histologic study was taken from the inner aspect of the thigh. Bone marrow samples were obtained by aspiration from the iliac crest or proximal femoral epiphysis. At approximately 6 months and again 3 years after the start of the experiment, laparotomies were performed and biopsies were taken of the organs previously mentioned. At death or sacrifice autopsies were performed as soon as feasible, usually within 3 hours of death.* Samples of tissue were obtained for histologic study and iron analysis from all the major organs at autopsy. All tissue samples were weighed within a short time of removal, the excess blood blotted off, and they were then wet-ashed with concentrated sulfuric acid. After digestion and dilution, the iron content was determined colorimetrically using the potassium thiocyanate (KSCN) method as described for serum iron determinations. Sections for histologic study were fixed in 10 per cent neutral formalin and Zenker's acetic solutions and stained with hematoxylin and eosin, and for iron with a modified Gomori stain,[60] counterstained with Mallory's basic fuschin. Selected sections were stained with Masson's connective tissue stain, methylene blue, periodic acid-Schiff, acid-fast, and Sudan IV stains.

RESULTS

Survival.—Of the 4 dogs which received saccharated iron oxide, two dogs (6 and 18) which had received 1.0 Gm. of iron per kilogram of weight were living and in good health at the time of sacrifice 81 and 84 months after the start of the experiment. One of the remaining animals that received Ferrivenin (17) died of intestinal obstruction due to biopsy-induced adhesions after 52 months, and the other dog (15) died in a laboratory accident 6 years after the onset of studies.

Three of the dogs receiving red cell transfusions died within a month of starting transfusions. The two animals that completed their course of transfusions were followed for 6 and 7 years. Dog 10 died in the same laboratory accident as Dog 15. Dog 20 was sacrificed after 84 months of the experiment. The ages of the dogs at death are unknown.

Reactions.—There was remarkably little reaction to single injections of large doses of saccharated iron oxide or to prolonged tissue iron accumulation. Weight was maintained or increased in all animals; appearance, vigor, and appetite remained good throughout the experiment. Only 3 immediate reactions of vomiting or diarrhea were seen in more than 150 injections of Ferrivenin. In one dog, accidental perivenous infiltration of the iron solution resulted in a 2 cm clean, flat based ulcer on the leg which healed completely within 10 days Fibrosis of the veins that occurred in most dogs toward the end of the course of injections was believed to be due more to the trauma of multiple venepunctures than to any sclerosing action of the iron solution

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*Two dogs (10 and 15) died at night when a steam pipe burst in their quarters; they were not autopsied until the next day when the accident was discovered. Considerable postmortem autolysis occurred in the tissues of these animals, but the distribution of iron and lack of fibrous reaction in the various tissues seemed in every way comparable to the findings in the other dogs.

Transfusions of washed red cells suspended in saline were poorly tolerated and led to the death of 3 dogs The exact cause of death is not known but premonitory signs of vomiting and diarrhea occurred shortly after completion of transfusions Two of the dogs (13 and 19) were later found dead in their cages, but in the third animal (Dog 12) the events leading to death were observed Despite normal temperature, pulse, and breath sounds, there was progression to an involuntary running reflex, paralysis of the forelegs, and finally rhythmic convulsions Adrenalin, oxygen administration, and phlebotomy were ineffective in reversing the reaction Neither hemoglobinuria nor anuria was found, incoagulability of the blood was not present, and blood samples cross matched with those of the donor dogs revealed no agglutination or hemolysis Furthermore, autopsy examination revealed only marked congestion of all organs with no cause of death determined

When whole blood preserved with 4 per cent sodium citrate was substituted for the A C D preserved, saline suspended, washed red cells no further untoward reactions were noted in 120 transfusions No evidence of gross hemolytic reactions was noted following any transfusion

Unrelated to the iron administration was the occurrence in Dog 17 of a severe upper respiratory infection thought to be distemper The infection was treated with penicillin and Aureomycin with complete recovery within 5 days This animal, following its second laparotomy, had a complete wound dehiscence with herniation of the bowel and spleen Early repair and vigorous antibiotic therapy resulted in prompt closure of the wound and complete healing, though the dog died several years later of intestinal obstruction due to peritoneal adhesions

Hematologic Response—Analysis of the red cell count, hemoglobin, and packed cell volume determinations reveals no marked variations from normal in the dogs given saccharated iron oxide Dog 6 is representative of this group (Fig 1) In all cases there was a moderate rise in the red count accompanied by a less distinct rise in hemoglobin and hematocrit 2 to 3 weeks after the initial administration of Ferrivenin A marked fall of all blood values was noted in Dog 18 toward the end of the iron injections and similar but less marked patterns were seen for Dogs 6 and 15 In all of these dogs complete restoration to normal was noted within 1 to 3 months after injections were completed Dog 17 showed little fall in any of the blood values during the injection period, but two months later had a definite decrease in all parameters with further accentuation at the time of an intercurrent bout of distemper Within a month, recovery was complete Subsequent determinations revealed only moderate fluctuations of blood counts Since these dogs had normal blood values initially, there was no reason to expect any marked rise in red cell elements from iron administration On the other hand, the fall noted in some instances shows a variable temporal relationship with the iron injections and it is impossible to determine with the data available the mechanism or causal relationship to iron administration.

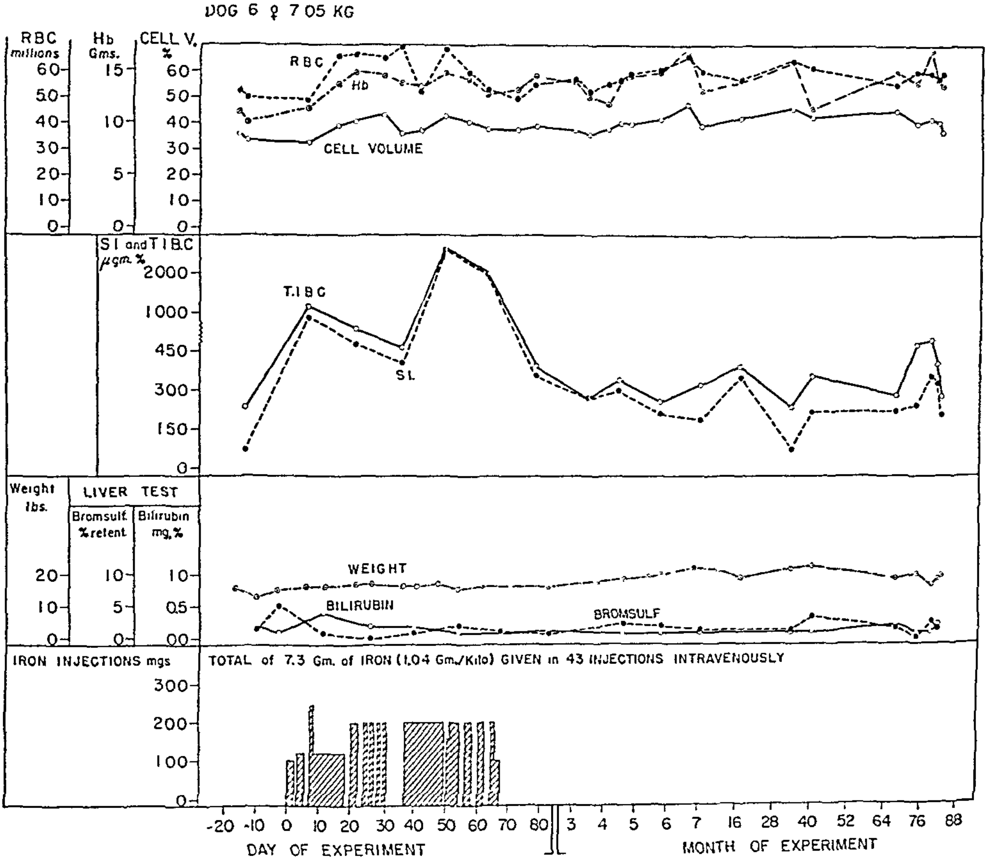

Fig. 1.—Effect of iron overload from injections of Ferrivenin on the blood counts, serum iron, body weight, and liver function of a dog observed 7 years. The amounts of iron injected intravenously are shown at the bottom of the chart.

A severe plethora was produced in all the transfused dogs. In the two dogs which received the greatest number of transfusions red cell counts reached levels greater than 15 million. Hemoglobin values were correspondingly elevated to peak values exceeding 26 Gm. per cent. Packed cell volumes were also elevated. These findings are shown graphically for Dog 10 in Fig. 2. Within a month following the last transfusion into Dog 10, the red cell count fell from 13 million to 6.5 million red cells per cubic millimeter and there were corresponding decreases in cell volume and hemoglobin values. A similar, but less well documented, fall to subnormal values occurred in Dog 20. There was no evidence of external blood loss; the anemia seemed most likely to be due to either a profound depression of the bone marrow secondary to prolonged transfusions, or an increased capacity for red cell breakdown by the reticuloendothelial system, or to a combination of both factors. A similar phenomenon was reported by Krumbhaar and Chanutin[14] though they noted its occurrence during the course of transfusions and found it to be unaffected by splenectomy.

Bone marrow aspirations were done on the dogs given saccharated iron oxide approximately one month after the course of injections. Except for slight hypocellularity of both myeloid and erythroid elements in Dogs 15 and 18 and large granular aggregates of hemosiderin in all animals, no abnormalities were noted. Enumeration of leukocytes, reticulocytes, and platelets was not done until after the completion of the period of intravenous iron injections. Intermittent eosinophilia as high as 20 per cent was observed in most animals and was thought to be related to intestinal parasitism. No other significant changes were obtained on differential leukocyte counts. Reticulocyte and platelet counts taken at infrequent intervals were normal throughout the period of observation.

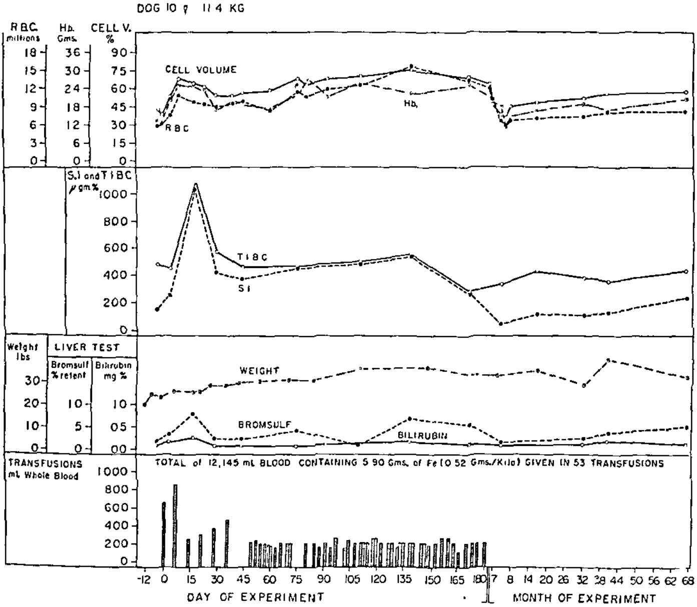

Fig. 2.—*Effect of iron overload from multiple transfusions on the blood counts, serum iron, body weight, and liver function of a dog observed 6 years. The amounts of blood transfused and the total iron content are shown at the bottom of the chart.*

Serum Iron Response.—Within a week after injections of saccharated iron oxide were begun serum iron values rose to between 400 and 1,000 µg per cent with approximately 80 per cent saturation.* A plateau was then maintained until the seventh week of injections when a further rise to peak values usually greater than 2,000 μg per cent with 97 to 100 per cent saturation was seen. Except for Dog 18, discontinuation of intravenous iron injections resulted in a prompt fall in serum iron, though per cent saturation remained almost 100 per cent in all animals. Normal serum iron and per cent saturation levels were reached about 6 months after injections were completed, and except for consistently subnormal values for both measurements at 33 months, erratic variations within the normal range occurred thereafter.

Of the tranfused animals, Dog 10 behaved more nearly like those injected with Ferrivenin. The initial marked rise in serum iron and per cent saturation was related to the early massive amounts of blood this animal received. However, when blood was given in smaller quantities at a slower rate, both transfused dogs had only moderate rises in serum iron though the iron binding capacity remained saturated for prolonged periods. Except for its last measured serum iron, Dog 10 maintained low normal values from the eighth month onward. Serum iron and per cent saturation reached a low ebb in Dog 20 at 30 months and subsequently fluctuated within the normal range until the final low serum iron value at sacrifice.

Acute changes in serum iron values were measured at intervals for 24 hours after the intravenous injection of 100 mg. of Ferrivenin into 3 dogs. Two of these animals had received iron injections for a month, though no iron had been given for two days before measurement of acute serum iron changes; the third dog had received no previous iron injections. Prompt rise to extremely high serum iron levels (3,000 to 7,500 μg per cent) with complete saturation of the transferrin occurred uniformly at 30 minutes, followed by a rapid fall at 2 hours and a return to approximately base-line values after 24 hours.

Certain aspects of the serum iron changes in these dogs are worthy of emphasis. First, there were no evidences of toxicity despite transient elevations of serum iron above 7,000 μg per cent and sustained elevations greater than 2,000 μg per cent. The upper limits of iron binding capacity in the dog are normally less than 500 μg per cent. Since even small amounts of unbound ionic iron produce marked toxic reactions,[61, 62] the extremely high levels observed without incident suggest (1) circulation of undissociated saccharated iron oxide, (2) marked elevation of the beta-1 globulin binding capacity, or (3) binding by other serum proteins. There is no evidence bearing directly on the latter two possibilities but presence of unsaturated iron binding capacity when serum iron levels were greater than 2,000 μg per cent strongly suggests circulation of undissociated saccharated iron oxide. Even more difficult to explain is the serum iron value of 1,032 μg per cent and 96 per cent saturation observed at 3 weeks in one of the transfused dogs (10). Unless contamination of the sample is postulated, one must assume circulation of ferritin or some hemoglobin breakdown product, since the serum iron method does not measure iron present as hemoglobin The plateau in serum iron after the initial high values from iron administration suggests the development of an increased clearing capacity that was later overwhelmed by the excessive iron load After injections were discontinued, gradual clearance was achieved, overcompensation by clearing elements might have been present to account for the low normal values seen at 6 months Somewhat surprising is the almost invariable desaturation of transferrin in the later periods of study despite the enormous tissue iron stores Compared to the situation in human hemochromatosis or transfusion hemosiderosis where complete saturation is the rule, the storage iron in the dogs' tissues appears to be bound more tightly and not in as free exchange with the serum iron compartment

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*Per cent saturation is the quotient of serum iron and total iron binding capacity $\left(\frac{SI}{TIBC}\right)$. The total iron binding capacity is defined here as the sum of serum iron and unsaturated iron binding capacity. See following discussion for possible explanations why the iron binding capacity is not saturated despite such high serum iron values.

Radioiron Studies—The absorption of oral doses of radioactive iron was measured in 3 dogs with large tissue iron stores Dog 18 was studied twice initially, 3 months after completion of its course of Ferrivenin injections, and again 6 years later Similar measurements were made once on Dog 6 about 6½ years after Ferrivenin injections and on Dog 20 approximately 5½ years after completion of the series of transfusions These values are tabulated (Table II)

TABLE II ABSORPTION OF ORAL DOSE OF 1 MG/KG OF RADIOIRON BY DOGS WITH IRON OVERLOAD

DOGS*	MONTHS AFTER LAST Fe ADMINISTRATION	PER CENT OF ORAL DOSE IN BLOOD	PER CENT OF ORAL DOSE IN FECES	PER CENT RECOVERY
18	3	1 7	98 2	99 9
	75	0 7	93 2	93 9
6	79	0 8	101 1	101 9
20	77	1 2	97 4	98 6

*Dogs 18 and 6 had received 1 Gm/Kg of iron as Ferrivenin Dog 20 had received 0 5 Gm/Kg of iron from multiple transfusions

Values for the absorption of comparable doses of orally administered radioactive iron in normal dogs vary from 0 8 to 8 8 per cent (average 5 8 per cent)[63] It appears from the figures that in these dogs with massive tissue iron deposits there was a low rate of iron absorption, but iron that was absorbed was quickly utilized for production of circulating hemoglobin

Utilization for hemoglobin synthesis of intravenously administered radioactive iron (2 4 to 7 4 mg) was measured in 3 dogs which had completed courses of saccharated iron oxide Dogs 15 and 18 showed radioiron utilization values of 30 and 31 per cent, well within the normal range[59] Dog 17, however, showed a greatly depressed iron utilization curve correlated most likely with the coincidental appearance of distemper Similar depression of iron utilization has been reported in dogs and human beings during febrile illnesses[59, 64, 65] If the curve for the dog with distemper is disregarded, the results of iron utilization studies on the other two animals suggest that despite the increased amounts of tissue iron, utilization of additional small amounts of intravenous iron was still within normal limits

Tests of Hepatic Function—Periodic evaluation of hepatic function was made by means of measurement of Bromsulphalein retention and serum bilirubin

TABLE III. TOTAL AND FRACTIONAL SERUM PROTEIN ANALYSES ON DOGS WITH IRON OVERLOAD

MONTHS AFTER START OF EXPERIMENT	DOG 6			DOG 20*			DOG 18			DOG 10			DOG 15			DOG 17		
	TOTAL PRO-TEIN	ALB.	GLOB.	TOTAL PRO-TEIN	ALB.	GLOB.	TOTAL PRO-TEIN	ALB.	GLOB.	TOTAL PRO-TEIN	ALB.	GLOB.	TOTAL PRO-TEIN	ALB.	GLOB.	TOTAL PRO-TEIN	ALB.	GLOB.
17	6.0	4.6	1.4	7.0	3.5	3.5	7.0	4.8	2.2	5.6	4.0	1.6	6.5	4.2	2.3	7.0	4.9	2.1
33	6.4	4.8	1.6	6.9	3.6	3.3	6.0	4.4	1.6	6.1	4.4	1.7	6.5	4.7	1.8	6.5	5.0	1.5
40	6.0	3.7	2.3	6.7	3.8	2.9	5.3	3.5	1.8	6.0	3.8	2.2	5.8	3.9	1.9	5.2	3.8	1.4
67	6.0	3.7	2.3	5.9	3.9	2.0	5.0	3.2	1.8	5.7	3.9	1.8	5.9	4.8	1.1	--	--	--
74	5.4	2.7	2.7	6.2	4.5	1.7	5.0	2.4	2.6	--	--	--	--	--	--	--	--	--
79	6.3	3.4	2.9	6.1	--	--	4.9	2.7	2.2	--	--	--	--	--	--	--	--	--
81	5.9	3.4	2.5	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
84	--	--	--	6.2	1.8	4.4	4.7	2.8	1.9	--	--	--	--	--	--	--	--	--
85	--	--	--	5.9	1.6	4.3	--	--	--	--	--	--	--	--	--	--	--	--

*The experiment on Dog 20 was begun 3 months after those on the other animals. Hence, the time of each sample is 3 months less than that shown in column one.

Dogs 6 and 18 had received iron as Ferrivenin in doses of 1.0 Gm./Kg.; Dogs 15 and 17 had received 0.5 Gm./Kg. of Ferrivenin; and Dogs 10 and 20 had received 0.5 Gm./Kg. of iron from multiple transfusions.

levels To these tests serum protein determinations were added 17 months after the start of iron administration Paper electrophoretic analyses of serum protein patterns were also determined during the last 18 months of the experiment

All dogs had normal Bromsulphalein retention (less than 12 per cent at 30 minutes)[66] except Dog 17 which had 22 4 per cent retention before any iron had been given, and Dog 18 which showed an isolated elevation of 13 7 per cent 5 weeks after the start of iron injections No pattern of even minor impairment of Bromsulphalein clearance could be discerned, nor were any differences noted between the animals given Ferrivenin and those transfused Likewise measurement of serum bilirubin failed to reveal abnormalities in any animal The indirect reacting fraction initially tended to be higher in the transfused animals in which large amounts of hemoglobin were being catabolized, but in no case were total bilirubin values greater than 0 5 mg /100 ml observed The most striking changes in serum proteins occurred in Dog 20 which showed a gradual fall in total proteins, a marked terminal fall in albumin and a rise in globulins with reversal of the albumin globulin ratio (Table III) Dog 18 likewise showed a progressive fall in total proteins due primarily to a fall in albumin but with no reversal of albumin globulin ratio Dog 17 likewise showed a progressive fall in total proteins in the three determinations performed No conclusive patterns were discernible in the remaining animals Analysis of paper electrophoretic separations of serum protein components confirmed the marked changes described above with the globulins increased mainly in the gamma fraction Less marked variations from normal were seen in the patterns of the other dogs

With the exception of the serum protein changes, all dogs showed no evidence of abnormal liver function whether they received saccharated iron oxide or transfusions The reversal of albumin globulin ratio demonstrated by Dog 20 is the only change suggestive of cirrhosis, the protein changes in the other animals are nonspecific Whether Dog 20's deviation from normal is related to the multiple transfusions or to hepatic iron storage cannot be answered unequivocally

Tests of Glucose Tolerance—Abnormal glucose tolerance associated with iron overload of the pancreas and/or the liver was evaluated by means of repeated fasting blood sugar determinations on all dogs and by oral glucose tolerance tests in 3 animals No elevation of fasting blood sugar was obtained on any dog throughout the experiment If any abnormality could be found, it was a mild hypoglycemic tendency Each animal showed blood sugar levels of less than 50 mg /100 ml at some time during its course Patterns of blood sugar values were similar in dogs injected with Ferrivenin and those which were transfused

Approximately 80 months after the start of iron administration, oral glucose tolerance tests were done on Dogs 6, 18, and 20, the results are shown graphically (Fig 3) Compared with normal dogs (shaded area), these animals showed no significant deviation except for Dog 20 In this dog, the same one which showed albumin globulin reversal, there was a moderate but sustained elevation of blood sugar throughout the 3½ year period of study While this curve reveals diminished glucose tolerance, it is not diagnostic of diabetes

Thus, with one possible exception, prolonged iron overload from transfusions or injected saccharated iron oxide failed to produce any laboratory evidence of diabetes mellitus.

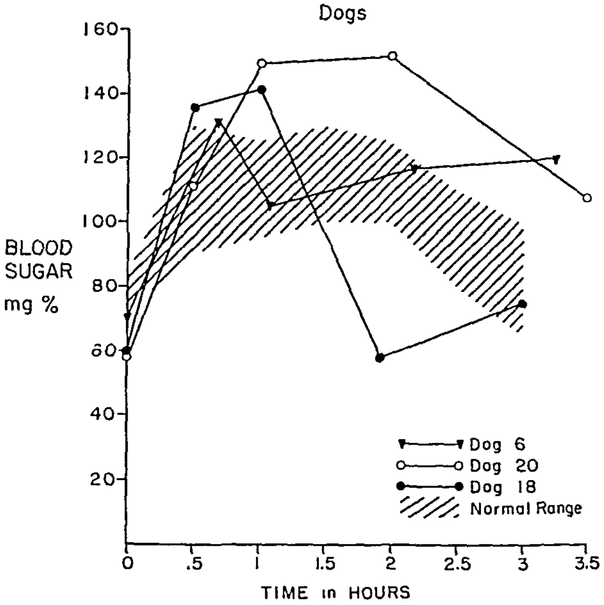


Fig. 3.—Oral glucose tolerance curves of three dogs with iron overload. Dogs 6 and 18 had received 1.0 Gm. per Kg. of iron as saccharated iron oxide, and Dog 20 had received 0.5 Gm. per Kg. of iron from multiple transfusions. The dose of glucose was 3 Gm. per Kg. of a 20 per cent solution. The normal range of glucose tolerance is shown in the shaded area.

Miscellaneous Observations.—Electrocardiograms were taken on Dogs 6, and 18 both 8 and 79 months after the beginning of Ferrivenin injections, and a single tracing was taken on Dog 20 at 76 months after the start of transfusions. The tracings were interpreted as being within normal limits.

The insidious development of blindness was observed in all animals beginning about 3 years after the start of iron administration and proceeding to complete loss of light perception. Ophthalmoscopic examination shortly before sacrifice revealed multiple small opacifications in the vitreous, perivascular sheathing of retinal vessels and degenerative changes of retinal nerve cell layers most marked at the periphery. These changes suggested that iron accumulation in the eye produced a retinopathy similar to siderosis bulbi and retinitis pigmentosa.

Two pups were born to Dog 6 approximately two years after the mother had received the 1 Gm./Kg. dose of saccharated iron oxide. These pups appeared to be normal, healthy animals and grew satisfactorily. At the time of sacrifice at 34 days of age both pups showed a mild anemia, and the large male pup had a serum iron level below normal. Despite the massive tissue iron concentration in the mother, there was no increase in the iron concentration of the pups' tissues. Histologic sections of selected tissues disclosed no abnormalities.

Tissue Iron Determinations.—Measurements of iron concentrations of tissues that could be readily biopsied were made on several occasions in order to quantitate the degree of iron accumulation and delineate any redistribution of iron that might occur. At autopsy, iron estimations of the biopsied organs and of additional tissues not accessible to biopsy were made. Estimates of the iron content of the major anatomical divisions of the body were calculated and by their summation an attempt was made to account for the iron administered parenterally.

All four dogs which received saccharated iron oxide underwent laparotomies before the start of iron injections, and the iron content of liver biopsies was determined. Likewise iron analyses of liver samples were performed initially on Dogs 12 and 13 which died early in the course of transfusions. No pretransfusion liver iron determinations were made on Dogs 10 and 20 which ultimately survived the complete course of transfusions. However, in all dogs biopsied, the initial iron concentration of the liver ranged from 0.081 to 0.343 mg./Gm. of wet tissue with an average of 0.205 mg./Gm. These figures agree well with those given for normal dogs by Bogniard and Whipple.[67]

Skin biopsies for iron analysis were obtained from the inner aspect of the thighs of all dogs which received Ferrivenin one to four weeks after their course of injections had been completed. These biopsies contained iron in concentrations varying from *0.160* to *0.386* mg./Gm., the normal average is 0.025 mg./Gm.

Laparotomies were again performed 6 months after beginning iron injections on each of the 4 dogs which had received Ferrivenin, and for the first time on Dog 10 which had just completed its course of transfusions. Tissue iron estimations were made on biopsies of liver, spleen, pancreas, kidney, muscle, skin, and mesenteric lymph node. These values and those of later biopsies are shown in Table IV.

Three years after the start of transfusions or Ferrivenin injections biopsies were repeated and samples of liver and spleen were analyzed for iron as before. No discernible pattern was evident in the changes of iron concentration of liver and spleen specimens from these repeated post iron biopsies. One animal, Dog 6, had consistently higher concentrations of iron in the liver than in the spleen, Dogs 18 and 10 showed the opposite pattern repeatedly, and Dogs 15 and 17 showed reversals of maximal iron concentrations in liver and spleen on successive determinations. Despite the difference noted, it is clear that iron given to these dogs accumulated in high concentrations in both spleen and liver reaching values as much as 50 to 75 times normal.

The concentration of iron in various tissues sampled at autopsy is given in Table V. Notable is the remarkable uniformity of iron values obtained from heart, brain, pancreas, and stomach in all dogs with or without excessive iron stores, emphasizing the absence of accumulation of iron in these organs. The moderate variations in iron concentration of lungs, adrenals, and mesenteric nodes and the marked fluctuations in liver, spleen, bone marrow, and peripancreatic lymph nodes undoubtedly reflect true differences in iron deposition rather than sampling errors. These figures emphasize the predominant localization of iron in organs with large reticuloendothelial components.

TABLE IV. IRON CONCENTRATION (MG./GM. WET WEIGHT) OF TISSUES OBTAINED AT BIOPSY OF DOGS WITH IRON OVERLOAD

TIME OF BIOPSY	DOG 6		DOG 18		DOG 20		DOG 10		DOG 15		DOG 17	
	LIVER	SPLEEN	LIVER	SPLEEN	LIVER	SPLEEN	LIVER	SPLEEN	LIVER	SPLEEN	LIVER	SPLEEN
Pre-iron control	0.81	---	0.343	---	---	---	---	---	0.222	---	0.223	---
Four months after iron	9.07	3.07	15.1	16.05	---	---	5.82	11.6	7.50	3.72	1.68	3.86
36 months after iron	14.8	6.98	8.04	13.0	10.1	12.45	4.3	4.86	5.55	11.4	6.82	4.14

TABLE V Iron Concentration (Mg./Gm. Wet Weight) of Tissues Obtained at Autopsy of Normal Dogs and Dogs With Iron Overload

TISSUES	DOG 6	DOG 18	DOG 20	DOG 10	DOG 15	DOG 17	NORMAL DOG (AVERAGE)
Liver	7.08	4.58	4.26	2.11	2.94	5.00	0.20
Spleen	5.23	6.90	14.46	4.28	3.58	11.6	0.24
Pancreas	0.05	0.04	0.05	0.11	0.07	0.13	0.06
Kidney	0.23	0.13	0.14	0.19	0.15	0.15	0.08
Heart	0.04	0.05	0.06	0.09	0.07	0.07	0.07
Lungs	0.22	0.14	0.20	0.36	0.36	0.26	0.25
Stomach	0.06	0.04	0.04		-		0.04
Mesenteric node	0.35	0.16	0.27	0.36	0.24		0.06
Peripancreatic node	18.65	11.8	5.34	--		3.36	-
Vertebral marrow	2.22	8.53	1.63	0.66	0.66	1.77	
Femoral marrow	4.04	2.46	0.98			-	0.22
Muscle	0.04	0.27	0.05	0.03	0.03	0.06	0.04
Abdominal skin	0.03	0.14	0.03	0.29	0.09	0.01	0.03
Brain	0.02	--	0.01	0.03	0.08	0.07	0.02
Eye	0.27	0.02	0.02	0.02	0.18		0.01
Thyroid	0.21	0.05	0.05	-			0.06
Adrenal	0.30	0.23	0.13	--			0.07

An attempt was made to estimate total carcass iron from the product of the organ concentrations and weight plus estimates of skin, muscle and bone marrow iron increments. To the carcass iron estimates were added the iron loss from repeated blood sampling, and in Dog 6 the iron loss due to the birth of pups. The sum of this accountable iron was compared to the amounts of iron given each dog. As seen in Fig. 4, 37 to 73 per cent of the injected iron could be accounted for by such a calculation. Though greatest recovery was found in the two dogs receiving the largest concentrations of iron as Ferrivenin, recovery of iron seemed independent of length of survival or type of iron given.

Some of the reasons for failure of complete recovery of administered iron can be enumerated. (1) There may be considerable error in estimating the iron content of muscle, skin, lymph nodes, and bone marrow since these organs cannot be easily weighed. (2) Sampling errors, despite duplicate measurements, in tissues with high iron content such as liver, spleen, and bone marrow are magnified in total organ iron estimates. (3) An unknown, but possibly appreciable loss of iron may have occurred via the gastrointestinal tract due to blood loss from intestinal parasites. Despite frequent tests for occult blood in the feces and repeated use of vermifuges, parasites were known to be present and were found at autopsy in two dogs. Loss of as little as 1 ml. of blood daily by this means would account for as much as 1.25 Gm. of iron during a 7 year period. (4) Loss of saccharated iron oxide in the urine during the period of tremendously elevated plasma iron levels might further account for considerable iron loss. Measurement of radioiron excretion acutely after intravenous injection of 100 mg. doses of Fe^{59} tagged Ferrivenin revealed approximately 1 per cent excretion in a normal dog and about 5 per cent excretion in a dog with massive iron overload. If one assumes an intermediate figure of 2 per cent for these animals an additional 100 to 200 mg. of iron might have been

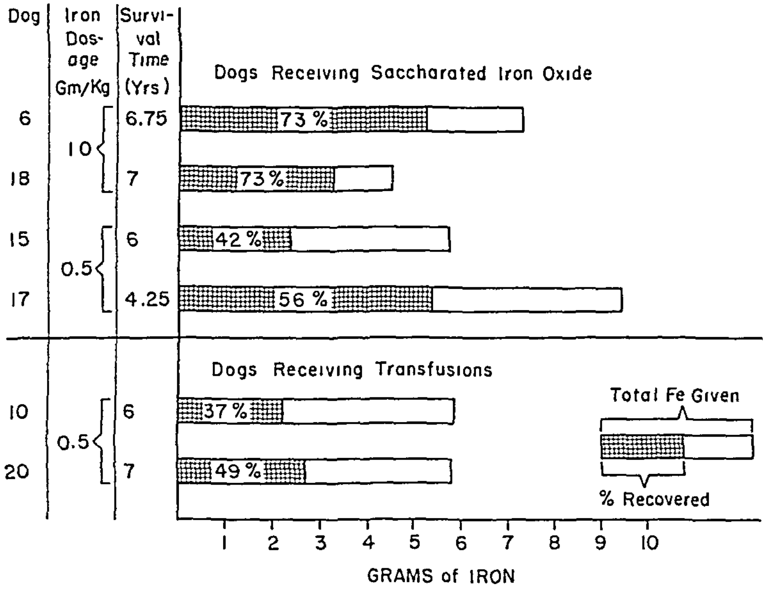


FIG. 4.—Estimate of the iron recovered at autopsy from the tissues of 6 dogs with iron overload, plotted as per cent of the total iron injected. The total amount of iron given to each dog is indicated by the scale at the bottom of the chart.

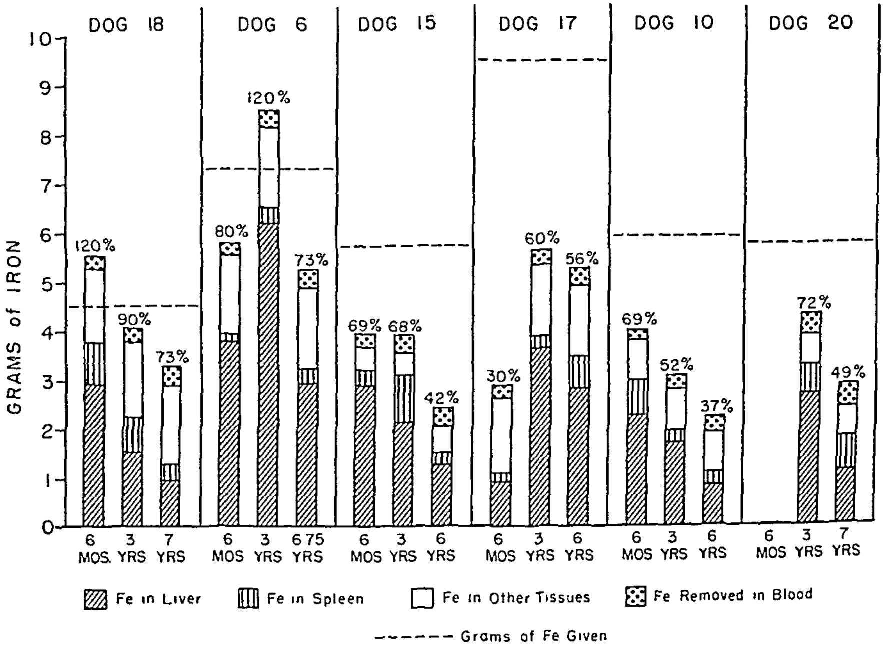


FIG. 5.—Shifts in body iron observed in 6 dogs with iron overload, estimated from the iron found in successive biopsy specimens and in the tissues at autopsy. The time of the biopsy or the autopsy is given at the bottom of each column. In each case, the column farthest to the right represents the autopsy data.

lost by this route. (5) Finally, increased excretion of iron through normal channels by these dogs with massive iron stores may have occurred. Such a phenomenon has been reported in iron heavy mice by Finch and associates.[68, 69] Since radioiron excretion studies were not performed on these animals, no answer can be given to this question. However, to account for the iron deficits noted by assuming increased excretion alone, one would have to postulate daily iron excretion of 0.5 to 2.6 mg. Excretion values corresponding to the lower figure have been reported for dogs with phenylhydrazine induced hemolytic anemia,[0] but a level of iron excretion high enough to explain the discrepancy between the iron administered and the iron recovered seems improbable.

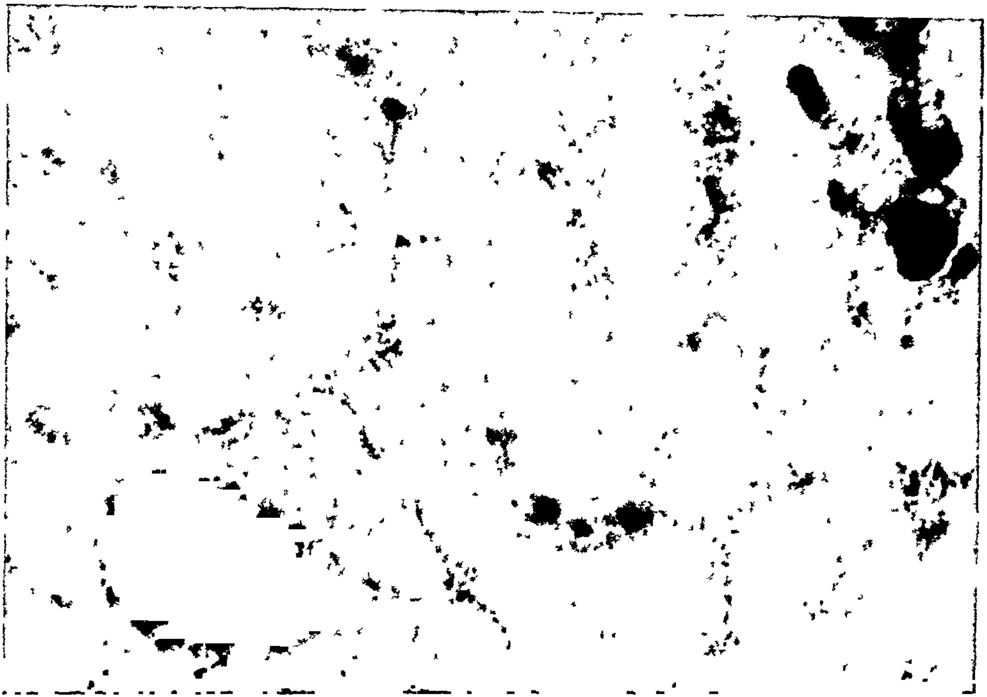
Furthermore, if a constant increased rate of iron excretion occurred it should be possible to detect it by comparing estimations of the total carcass iron at the time of the 6 and 36 month biopsies with that at autopsy. In order to make this comparison, certain assumptions are necessary, namely, (1) the weights of various organs remained constant throughout the experiment and were the same as at autopsy, (2) the iron content of organs other than liver and spleen remained the same as found at autopsy and (3) correction was made for iron withdrawn by blood sampling at the various times. Then the iron content of liver and spleen was calculated from the weights of these organs multiplied by their iron concentrations at biopsy or autopsy. To these figures were added the iron content of other tissues and the increment due to blood sampling, and this total was compared to the amount of iron given during the course of the experiment. The results of such an analysis are presented in Fig. 5. In Dogs 18, 15, 10, and 20 a progressive decline in liver iron was observed paralleled by a fall in estimated total carcass iron recovery. Iron content of the spleen decreased progressively in Dogs 18 and 10, increased in Dogs 17 and 20, and oscillated in Dogs 6 and 15. By these admittedly crude calculations, total body iron estimated from biopsy values exceeded the amounts given by 20 per cent on two occasions. Thus, while recovery of iron at autopsy in all but one dog was lower than the estimated recovery earlier in the experiment, the evidence for a constant increased excretory loss is meager.

Histologic Observations.—Throughout the course of the study, an effort was made to evaluate the sites of deposition of iron given parenterally and to delineate the histologic pattern of reaction to the increased tissue iron by repeated biopsies of accessible tissues and by extensive sampling at autopsy.

Control biopsies. The initial histologic sections of the dogs' tissues obtained at laparotomy before administration of iron revealed no abnormalities in the liver, spleen, pancreas, mesenteric lymph node, skeletal muscle, and skin. In none of the dogs was there any evidence of fibrosis or cirrhosis either by hematoxylin and eosin or connective tissue stains. Iron stains of these various tissue sections revealed a small amount of iron in the macrophages of the spleen and liver and rarely in the macrophages about the central sinusoids of the mesenteric lymph nodes. No evidence of hemofuscin was found in any section.

Post iron biopsies. Approximately one month after completion of Ferrivenin injections, skin biopsies were obtained on each of the 4 dogs. Iron deposition confined to the upper layers of the dermis, especially around the hair follicles, was the only noteworthy finding.

Fig. 6



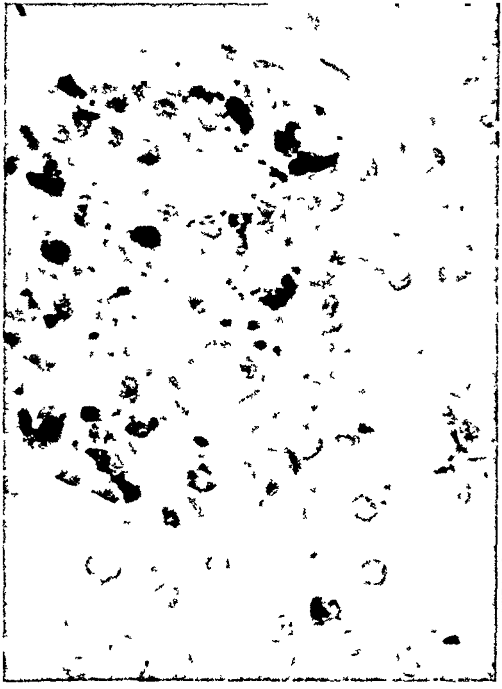


Fig 7

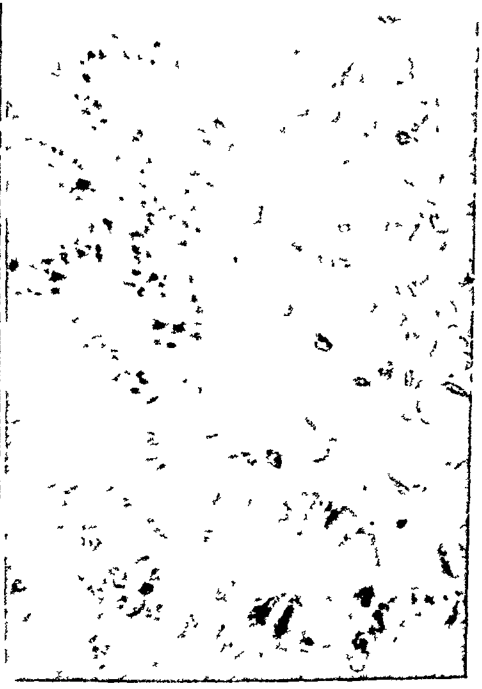


Fig 8

Fig 6 —Liver of Dog 10 one month after completion of transfusions There are large aggregates of granules that react positively for iron (black in photograph) in clumps of macrophages in the sinusoids and finer deposits along the sinusoidal endothelium and within the hepatic cells There is no fibrosis or hemofuscin Gomori's stain (Magnification ×595)

Fig 7 —Kidney of Dog 6 four months after 1 0 Gm per Kg of Ferrivenin Iron positive granules are present in endothelial cells of capillaries and in macrophages caught in the glomerular tuft. Gomori's stain (Magnification ×595)

Fig 8 —Kidney of Dog 10 one month after transfusions Iron positive granules are present in tubular epithelium but not in the glomeruli Gomori's stain. (Magnification ×595)

A second laparotomy was done 4 months after Ferrivenin injections were completed (Dogs 6, 18, 15, 17) and additional biopsy specimens for histologic examination were obtained. In Dog 10 a laparotomy with biopsy of the aforementioned organs was performed approximately one month after completion of transfusions. Except for the sections of the kidney, the histologic appearance of the various organs was identical for the various dogs and was independent of the dose of iron whether administered as Ferrivenin or transfused blood. Sections stained with hematoxylin (H) and eosin (E) revealed marked pigmentation of the liver, spleen, and mesenteric lymph nodes, considerably less pigment in pancreas, kidney, and skin, and practically no pigment in the skeletal muscle. There was no evidence of increased fibrous tissue in the liver, pancreas, or other organs when stained with either H and E or Masson's stains and there were absolutely no cirrhotic changes. Iron stains of the liver revealed hemosiderin to be deposited in coarse granules aggregated into clumps with focal distention of sinusoids and deposits in the portal triads. In addition to these larger deposits there was a diffuse, fine granular deposition of iron in the hepatic parenchymal cells distributed uniformly throughout the lobule and in individual Kupffer cells (Fig. 6). Smaller amounts of iron were seen in the endothelium of the blood vessels, but no iron was found in the bile ducts. In the spleen, the iron was found in largest quantities in the macrophages of the sinusoids and to a lesser extent in the capsule, trabeculae, and beneath the endothelium of the blood vessels. No iron was seen in Malpighian corpuscles. Iron in the kidneys of the dogs given Ferrivenin (Fig. 7) was confined to a moderate amount in the glomerular capillary tufts with only a faint blue shade and rare, fine granules in the renal tubules. In contrast, the transfused dog showed small amounts of iron as fine blue granules in the proximal convoluted tubules with practically no iron seen in the glomeruli (Fig. 8). The pancreas in both groups revealed only small amounts of iron confined to macrophages and the endothelial layers of the blood vessels. There was no iron deposition in the acinous or islet epithelial cells, and no evidence of other pigment suggestive of hemofuscin. Mesenteric lymph nodes showed large quantities of iron in the macrophages of the central sinusoids with a less marked pigmentation of the peripheral macrophages. Iron in the skin was confined to macrophages in the dermis especially those around hair follicles, and was increased in amount compared to that seen in the skin biopsy sections taken 3 months previously. There was no iron or other pigmentation of the epidermis. The only iron seen in skeletal muscle sections was a faint blue cast to the capillary endothelium in one dog. No hemofuscin was seen in any of the sections.

Laparotomies, with biopsies of abdominal organs previously described, were performed on all 6 dogs approximately 3 years after completing iron administration. Histologic appearance of the tissues was identical to that seen at autopsy and a single description will suffice.

Autopsies. Gross examination revealed no free fluid in the pleural, pericardial, or peritoneal cavities of any of the dogs. Fibrous adhesions of the peritoneum were seen in most dogs at sites of old anterior abdominal incisions and organ biopsies. A distinct, rust brown color was noted in the lymph nodes, pancreas, spleen, and kidney; the liver was a dark mahogany brown. In the two dogs with post mortem autolysis, a diffuse, shiny, black pigmentation, presumably due to iron sulfide, was present in all abdominal organs and with greatest intensity in the intestines. In two dogs Ascaris infestation was demonstrated in the upper portion of the small intestine. A single laminated gallstone was found in the gall bladder of Dog 6. No abnormalities of heart, lungs, great vessels, brain, or other organs were apparent grossly.

Microscopic appearance of the tissues of all dogs is described in composite fashion except in those cases where distinctive changes were present. The basic architecture of the liver was normal (Fig. 9) with no evidence of cirrhosis or even increased fibrous tissue in any animal. The iron was present principally in large compact clumps of heavily laden macrophages (siderophages) which were present at random sites in the sinusoids and in large masses in the portal triads. Individual Kupffer cells also contained moderate amounts of iron. There was only the faintest hint of fine hemosiderin granules in the liver parenchymal